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(54) **THROMBOPOIETIC COMPOUNDS**

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(57) **ABSTRACT**

The invention relates to the field of compounds, especially peptides or polypeptides, that have thrombopoietic activity. The peptides and polypeptides of the invention may be used to increase platelets or platelet precursors (e.g., megakaryocytes) in a mammal.

8 Claims, 7 Drawing Sheets

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        ATGGACAAAACTCACACATGTCCACCTTGTCCAGCTCCGGAACTCCTGGGGGGACCGTCA
  1     ---------+---------+---------+---------+---------+---------+ 60
        TACCTGTTTTGAGTGTGTACAGGTGGAACAGGTCGAGGCCTTGAGGACCCCCCTGGCAGT

a       M  D  K  T  H  T  C  P  P  C  P  A  P  E  L  L  G  G  P  S  -

        GTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC
 61     ---------+---------+---------+---------+---------+---------+ 120
        CAGAAGGAGAAGGGGGGTTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAG

a       V  F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P  E  V  -

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121     ---------+---------+---------+---------+---------+---------+ 180
        TGTACGCACCACCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCAC

a       T  C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V  -

        GACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACG
181     ---------+---------+---------+---------+---------+---------+ 240
        CTGCCGCACCTCCACGTATTACGGTTCTGTTTCGGCGCCCTCCTCGTCATGTTGTCGTGC

a       D  G  V  E  V  H  N  A  K  T  K  P  R  E  E  Q  Y  N  S  T  -

        TACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC
241     ---------+---------+---------+---------+---------+---------+ 300
        ATGGCACACCAGTCGCAGGAGTGGCAGGACGTGGTCCTGACCGACTTACCGTTCCTCATG

a       Y  R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G  K  E  Y  -

        AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCC
301     ---------+---------+---------+---------+---------+---------+ 360
        TTCACGTTCCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGG

a       K  C  K  V  S  N  K  A  L  P  A  P  I  E  K  T  I  S  K  A  -

        AAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACC
361     ---------+---------+---------+---------+---------+---------+ 420
        TTTCCCGTCGGGGCTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGG

a       K  G  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  D  E  L  T  -

        AAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG
421     ---------+---------+---------+---------+---------+---------+ 480
        TTCTTGGTCCAGTCGGACTGGACGGACCAGTTTCCGAAGATAGGGTCGCTGTAGCGGCAC

a       K  N  Q  V  S  L  T  C  L  V  K  G  F  Y  P  S  D  I  A  V  -

        GAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGAC
481     ---------+---------+---------+---------+---------+---------+ 540
        CTCACCCTCTCGTTACCCGTCGGCCTCTTGTTGATGTTCTGGTGCGGAGGGCACGACCTG
```

FIG. 1A

```
a        E  W  E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P  V  L  D   -

         TCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAG
    541  ---------+---------+---------+---------+---------+---------+ 600
         AGGCTGCCGAGGAAGAAGGAGATGTCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTC

a        S  D  G  S  F  F  L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q   -

         GGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAG
    601  ---------+---------+---------+---------+---------+---------+ 660
         CCCTTGCAGAAGAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTC

a        G  N  V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K   -

         AGCCTCTCCCTGTCTCCGGGTAAA
    661  ---------+---------+---- 684
         TCGGAGAGGGACAGAGGCCCATTT

a        S  L  S  L  S  P  G  K
```

FIG. 1B

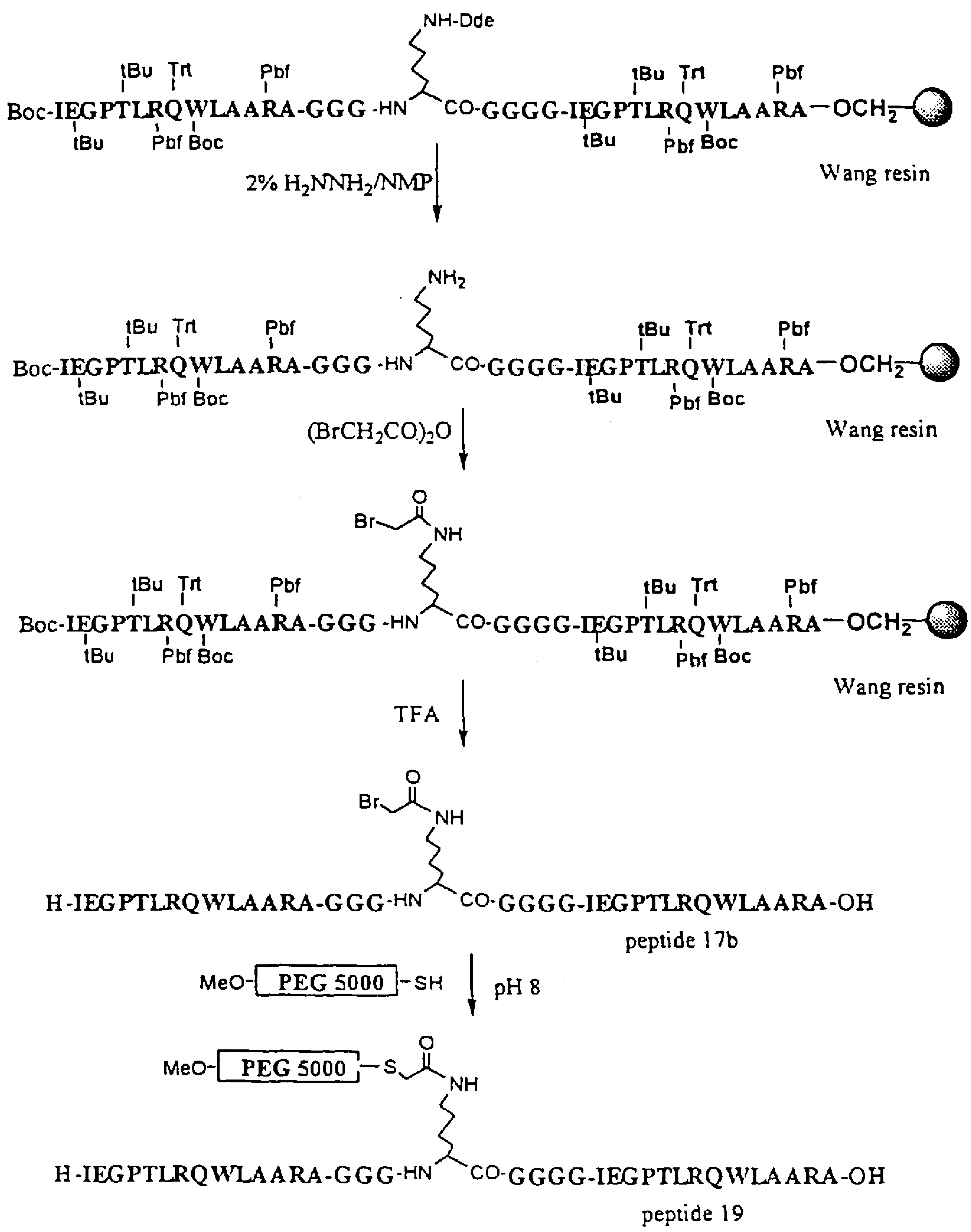
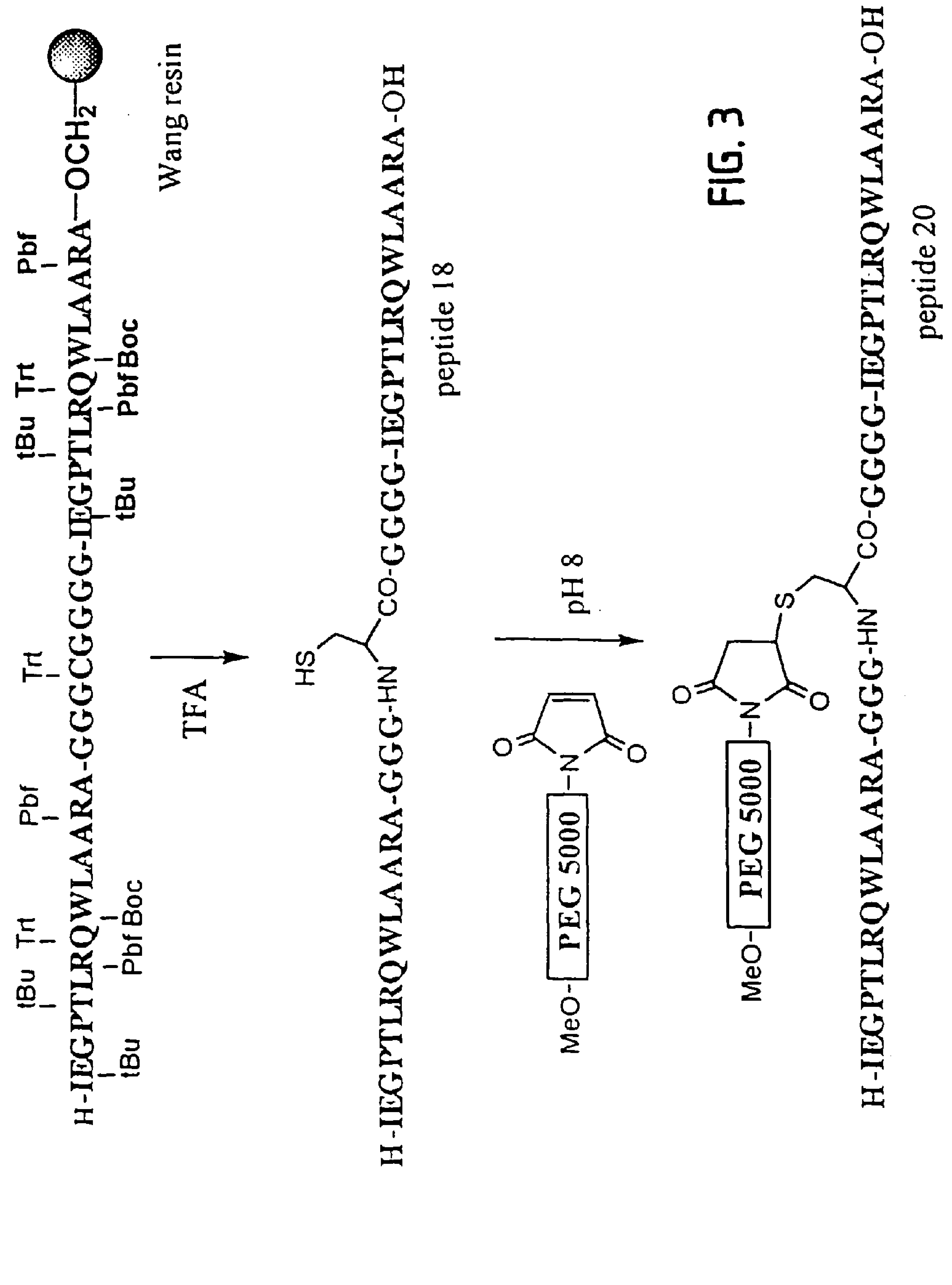

NH-Dde
tBu Trt Pbf
Boc-IEGPTLRQWLAARA-GGG-HN CO-GGGG-IEGPTLRQWLAARA—OCH2
tBu Pbf Boc
Wang resin
2% H2NNH2/NMP
NH2
(BrCH2CO)2O
Br
NH
TFA
H-IEGPTLRQWLAARA-GGG-HN CO-GGGG-IEGPTLRQWLAARA-OH
peptide 17b
MeO- PEG 5000 -SH
pH 8
peptide 19

FIG. 2



H-IEGPTLRQWLAARA-GGCGGG-IEGPTLRQWLAARA—OCH2
tBu
Trt
Pbf
Pbf Boc
Wang resin
TFA
HS
H-IEGPTLRQWLAARA-GGG-HN
CO-GGGG-IEGPTLRQWLAARA-OH
peptide 18
pH 8
MeO-
PEG 5000
S
CO-GGGG-IEGPTLRQWLAARA-OH
H-IEGPTLRQWLAARA-GGG-HN
peptide 20

FIG. 3

FIG. 4

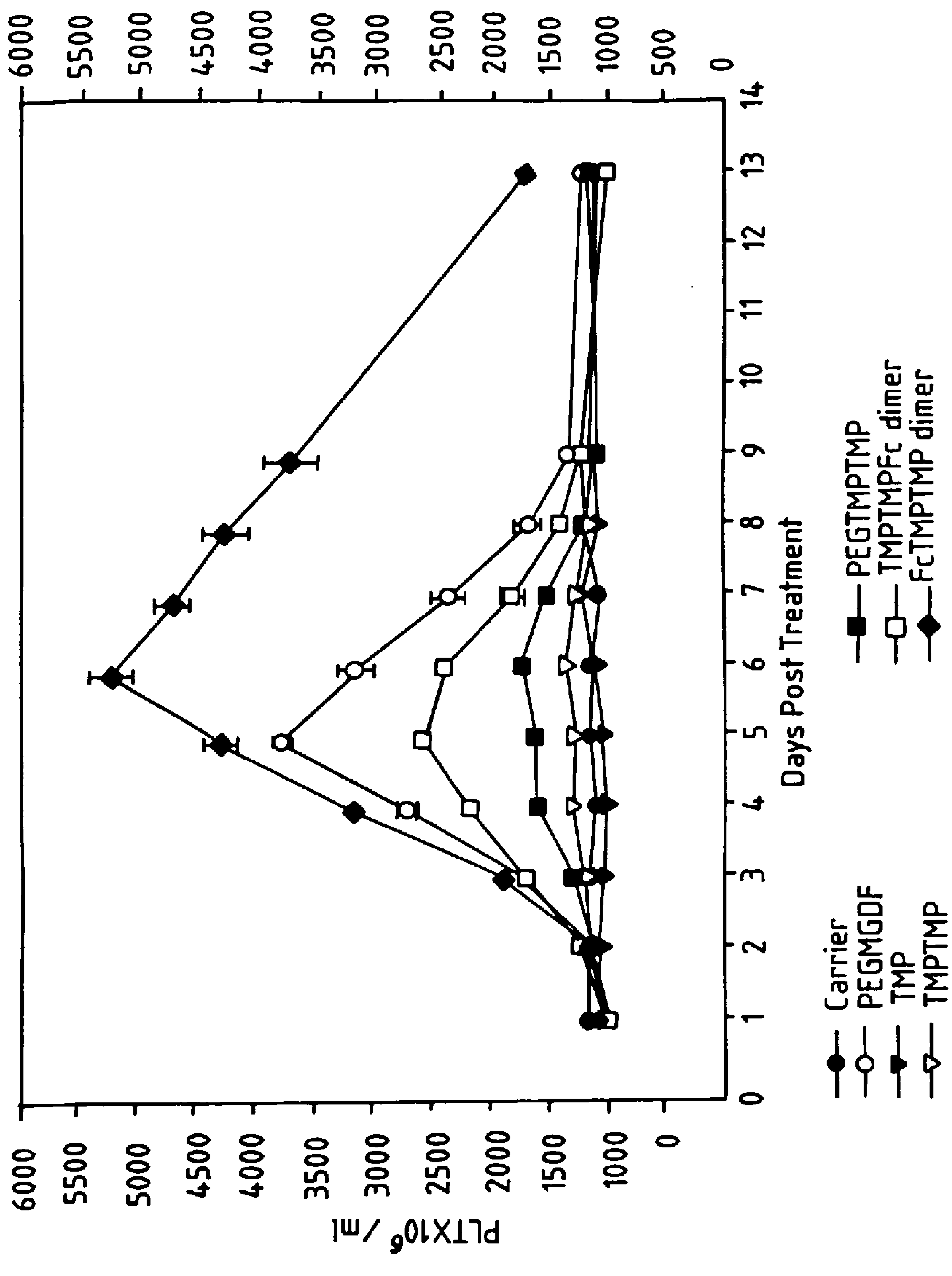

PLTX10⁶/ml
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Treatment
Carrier
PEGMGDF
TMP
TMPTMP
PEGTMPTMP
TMPTMPFc dimer
FcTMPTMP dimer

FIG. 5

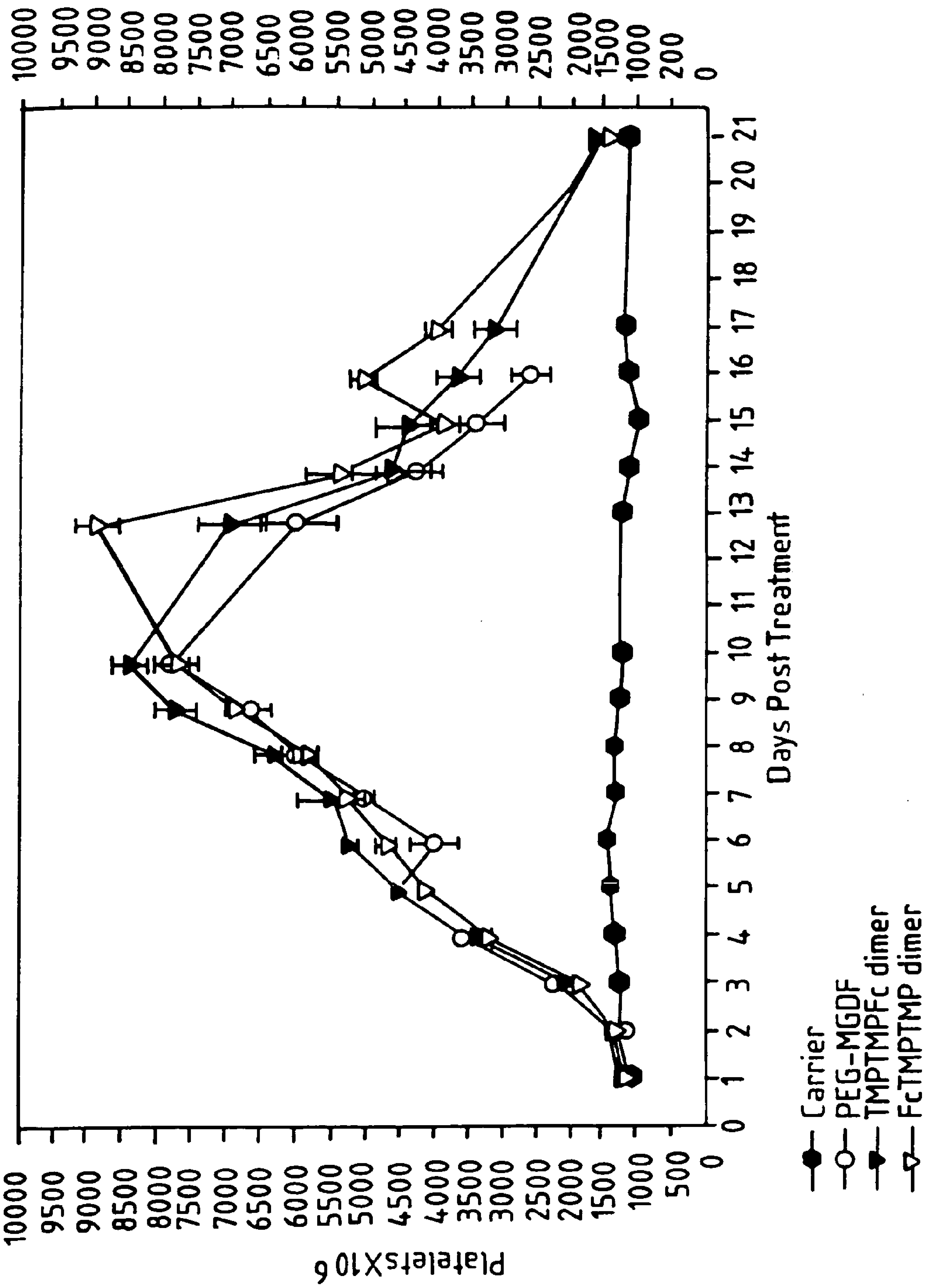

PlateletsX10 6
Days Post Treatment
Carrier
PEG-MGDF
TMPTMPFc dimer
FcTMPTMP dimer

FIG. 6A

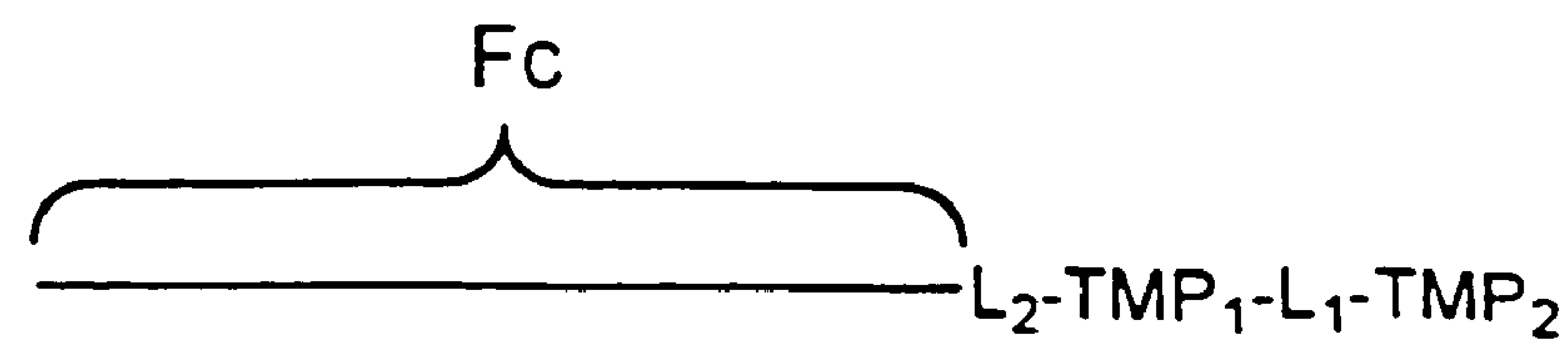


FIG. 6B

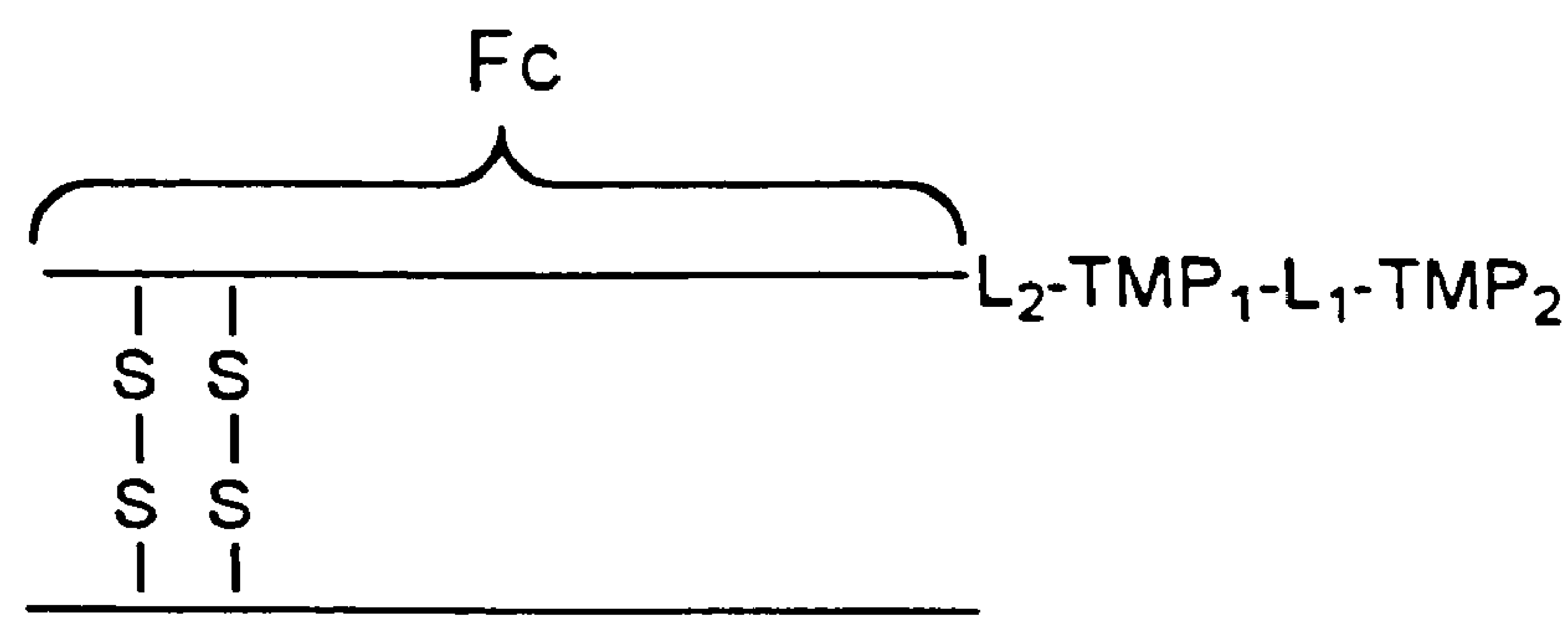
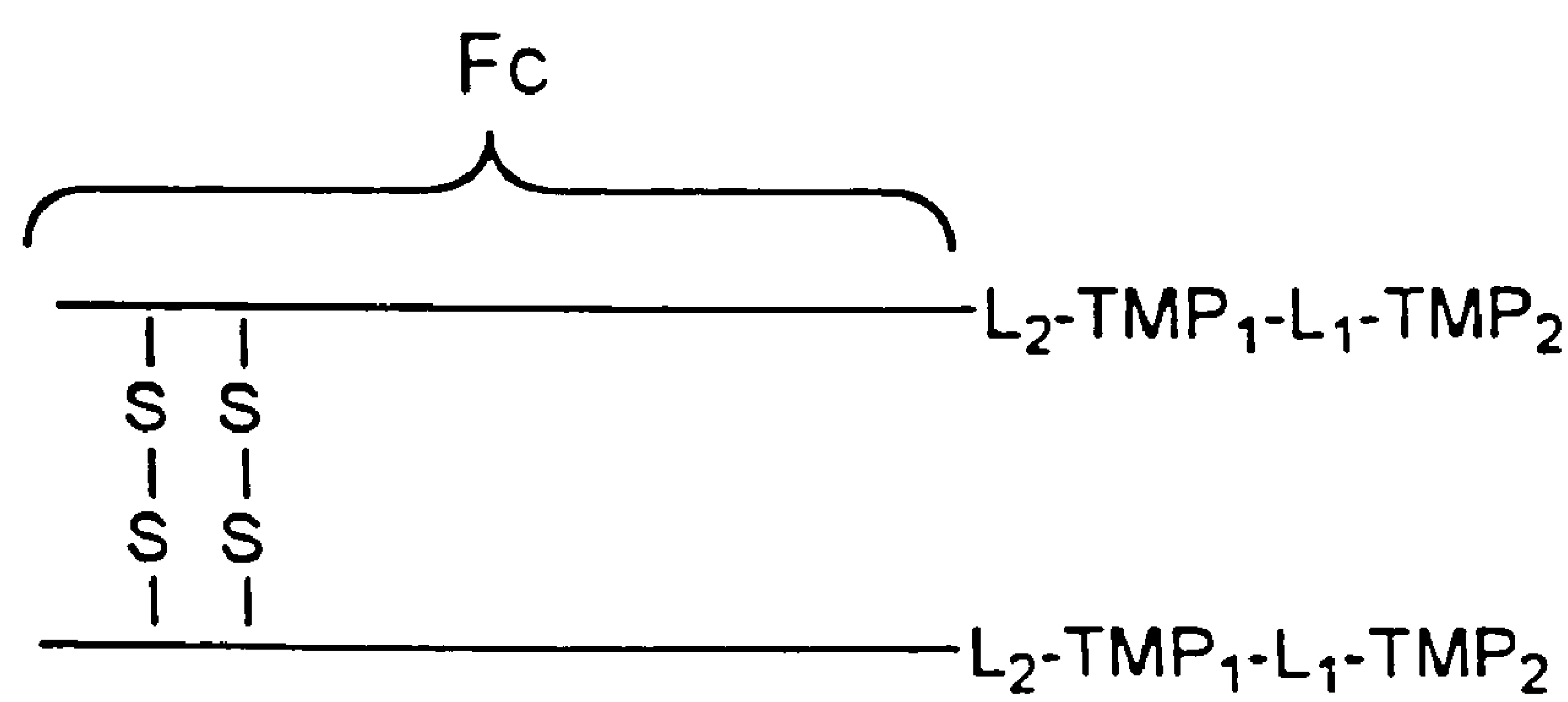


FIG. 6C



THROMBOPOIETIC COMPOUNDS

This application is a divisional application of U.S. patent application Ser. No. 09/422,838, which was filed Oct. 22, 1999, which in turn claims benefit under 35 U.S.C. §119 of U.S. Provisional Patent Application Application Ser. No. 60/105,348, which was filed Oct. 23, 1998, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

Generally, the invention relates to the field of compounds, especially peptides and polypeptides, that have thrombopoietic activity. The compounds of the invention may be used to increase production of platelets or platelet precursors (e.g., megakaryocytes) in a mammal.

BACKGROUND OF THE INVENTION

This invention relates to compounds, especially peptides, that have the ability to stimulate in vitro and in vivo production of platelets and their precursor cells such as megakaryocytes. Before discussing the nature of the inventive compounds, the following is provided as a background regarding two proteins that have thrombopoietic activity: thrombopoietin (TPO) and megakaryocyte growth and development factor (MGDF).

The cloning of endogenous thrombopoietin (TPO) (Lok et al., Nature 369:568-571 (1994); Bartley et al., Cell 77:1117-1124 (1994); Kuter et al., Proc. Natl. Acad. Sci. USA 91:11104-11108 (1994); de Sauvage et al., Nature 369:533-538 (1994); Kato et al., Journal of Biochemistry 119:229-236 (1995); Chang et al., Journal of Biological Chemistry 270: 511-514 (1995)) has rapidly increased our understanding of megakaryopoiesis (megakaryocyte production) and thrombopoiesis (platelet production).

Endogenous human TPO, a 60 to 70 kDa glycosylated protein primarily produced in the liver and kidney, consists of 332 amino acids (Bartley et al., Cell 77:1117-1124 (1994); Chang et al., Journal of Biological Chemistry 270:511-514 (1995)). The protein is highly conserved between different species, and has 23% homology with human erythropoietin (Gurney et al., Blood 85:981-988 (1995)) in the amino terminus (amino acids 1 to 172) (Bartley et al., Cell 77:1117-1124 (1994)). Endogenous TPO has been shown to possess all of the characteristics of the key biological regulator of thrombopoiesis. Its in vitro actions include specific induction of megakaryocyte colonies from both purified murine hematopoietic stem cells (Zeigler et al., Blood 84:4045-4052 (1994)) and human $CD34^+$ cells (Lok et al., Nature 369:568-571 (1994); Rasko et al., Stem Cells 15:3342 (1997)), the generation of megakaryocytes with increased ploidy (Broudy et al., Blood 85:402-413 (1995)), and the induction of terminal megakaryocyte maturation and platelet production (Zeigler et al., Blood 84:4045-4052 (1994); Choi et al., Blood 85:402-413 (1995)). Conversely, synthetic antisense oligodeoxynucleotides to the TPO receptor (c-Mpl) significantly inhibit the colony-forming ability of megakaryocyte progenitors (Methia et al., Blood 82:1395-1401 (1993)). Moreover, c-Mpl knock-out mice are severely thrombocytopenic and deficient in megakaryocytes (Alexander et al., Blood 87:2162-2170 (1996)).

Recombinant human MGDF (rHuMGDF, Amgen Inc., Thousand Oaks, Calif.) is another thrombopoietic polypeptide related to TPO. It is produced using *E. coli* transformed with a plasmid containing cDNA encoding a truncated protein encompassing the amino-terminal receptor-binding domain of human TPO (Ulich et al., Blood 86:971-976 (1995)). The polypeptide is extracted, refolded, and purified, and a poly[ethylene glycol] (PEG) moiety is covalently attached to the amino terminus. The resulting molecule is referred to herein as PEG-rHuMGDF or MGDF for short.

Various studies using animal models (Ulich, T. R. et al., Blood 86:971-976 (1995); Hokom, M. M. et al., Blood 86:4486-4492 (1995)) have clearly demonstrated the therapeutic efficacies of TPO and MGDF in bone marrow transplantation and in the treatment of thrombocytopenia, a condition that often results from chemotherapy or radiation therapy. Preliminary data in humans have confirmed the utility of MGDF in elevating platelet counts in various settings. (Basser et al., Lancet 348:1279-81 (1996); Kato et al., Journal of Biochemistry 119:229-236 (1995); Ulich et al., Blood 86:971-976 (1995)). MGDF might be used to enhance the platelet donation process, since administration of MGDF increases circulating platelet counts to about three-fold the original value in healthy platelet donors.

TPO and MGDF exert their action through binding to the c-Mpl receptor which is expressed primarily on the surface of certain hematopoietic cells, such as megakaryocytes, platelets, $CD34^+$ cells and primitive progenitor cells (Debili, N. et al., Blood 85:391-401 (1995); de Sauvage, F. J. et al, Nature 369:533-538 (1994); Bartley, T. D., et al., Cell 77:1117-1124 (1994); Lok, S. et al., Nature 369: 565-8 (1994)). Like most receptors for interleukins and protein hormones, c-Mpl belongs to the class I cytokine receptor superfamily (Vigon, I. et al., Proc. Natl. Acad. Sci. USA 89:5640-5644 (1992)). Activation of this class of receptors involves ligand-binding induced receptor homodimerization which in turn triggers the cascade of signal transducing events.

In general, the interaction of a protein ligand with its receptor often takes place at a relatively large interface. However, as demonstrated in the case of human growth hormone bound to its receptor, only a few key residues at the interface actually contribute to most of the binding energy (Clackson, T. et al., Science 267:383-386 (1995)). This and the fact that the bulk of the remaining protein ligand serves only to display the binding epitopes in the right topology makes it possible to find active ligands of much smaller size.

In an effort toward this, the phage peptide library display system has emerged as a powerful technique in identifying small peptide mimetics of large protein ligands (Scott, J. K. et al., Science 249:386 (1990); Devlin, J. J. et al., Science 249: 404 (1990)). By using this technique, small peptide molecules that act as agonists of the c-Mpl receptor were discovered (Cwirla, S. E. et al., Science 276:1696-1699 (1997)).

In such a study, random small peptide sequences displayed as fusions to the coat proteins of filamentous phage were affinity-eluted against the antibody-immobilized extracellular domain of c-Mpl and the retained phages were enriched for a second round of affinity purification. This binding selection and repropagation process was repeated many times to enrich the pool of tighter binders. As a result, two families of c-Mpl-binding peptides, unrelated to each other in their sequences, were first identified. Mutagenesis libraries were then created to further optimize the best binders, which finally led to the isolation of a very active peptide with an IC_{50}=2 nM and an EC_{50}=400 nM (Cwirla, S. E. et al., Science 276:1696-1699 (1997)). This 14-residue peptide, designated as a TMP (for TPO Mimetic Peptide), has no apparent sequence homology to TPO or MGDF. The structure of this TMP compound is as follows:

```
Ile Glu Gly Pro Thr Leu Arg Gln      SEQ ID NO: 1
Trp Leu Ala Ala Arg Ala
```

or

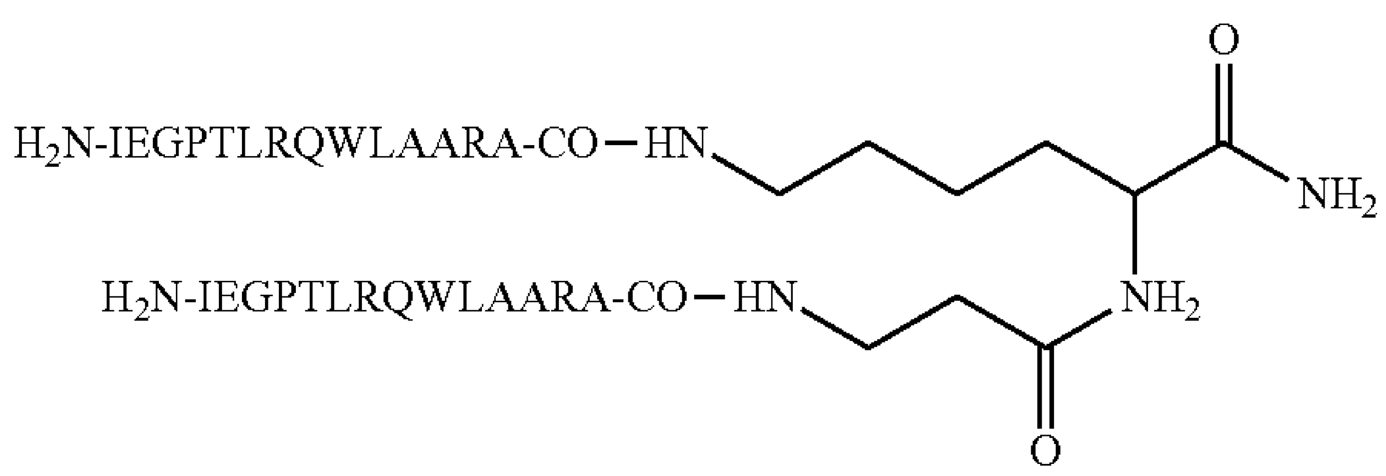
```
IEGPTLRQWLAARA
```

using single letter amino acid abbreviations.

Previously, in a similar study on EPO mimetic peptides, an EPO mimetic peptide (EMP) was discovered using the same technique (Wrighton, N. C. et al., Science 273:458463 (1996)), and was found to act as a dimer in binding to the EPO receptor (EPOR). The $(ligand)_2/(receptor)_2$ complex thus formed had a C2 symmetry according to X-ray crystallographic data (Livnah, O. et al., Science 273:464-471 (1996)). Based on this structural information, a covalently linked dimer of EMP in which the C-termini of two EMP monomers were crosslinked with a flexible spacer was designed and found to have greatly enhanced binding as well as in vitro/in vivo bioactivity (Wrighton, N. C., et al., Nature Biotechnology 15:1261-1265 (1997)).

A similar C-terminal dimerization strategy was applied to the TPO mimetic peptide (TMP) (Cwirla, S. E. et al., Science 276:1696-1699 (1997)). It was found that a C-terminally linked dimer (C-C link) of TMP had an improved binding affinity of 0.5 nM and a remarkably increased in vitro activity (EC_{50}=0.1 nM) in cell proliferation assays (Cwirla, S. E. et al., Science 276:1696-1699 (1997)). The structure of this TMP C-C dimer is shown below:

(SEQ ID NO: 2)



In another aspect of the present invention, the tandem dimers may be further attached to one or more moieties that are derived from immunoglobulin proteins, referred to generally as the Fc region of such immunoglobulins. The resulting compounds are referred to as Fc fusions of TMP tandem dimers.

The following is a brief background section relating to the Fc regions of antibodies that are useful in connection with some of the present compounds.

Antibodies comprise two functionally independent parts, a variable domain known as "Fab", which binds antigen, and a constant domain, known as "Fc" which provides the link to effector functions such as complement fixation or phagocytosis. The Fc portion of an immunoglobulin has a long plasma half-life, whereas the Fab is short-lived. (Capon, et al., Nature 337:525-531 (1989)).

Therapeutic protein products have been constructed using the Fc domain to attempt to provide longer half-life or to incorporate functions such as Fc receptor binding, protein A binding, complement fixation, and placental transfer which all reside in the Fc region of immunoglobulins (Capon, et al., Nature 337:525-531 (1989)). For example, the Fc region of an IgG1 antibody has been fused to CD30-L, a molecule which binds CD30 receptors expressed on Hodgkin's Disease tumor cells, anaplastic lymphoma cells, T-cell leukemia cells and other malignant cell types. See, U.S. Pat. No. 5,480,981. IL-10, an anti-inflammatory and antirejection agent has been fused to murine Fcγ2a in order to increase the cytokine's short circulating half-life (Zheng, X. et al., The Journal of Immunology, 154: 5590-5600 (1995)). Studies have also evaluated the use of tumor necrosis factor receptor linked with the Fc protein of human IgG1 to treat patients with septic shock (Fisher, C. et al., N. Engl. J. Med., 334: 1697-1702 (1996); Van Zee, K. et al., The Journal of Immunology, 156: 2221-2230 (1996)). Fc has also been fused with CD4 receptor to produce a therapeutic protein for treatment of AIDS. See, Capon et al., Nature, 337:525-531 (1989). In addition, interleukin 2 has been fused to the Fc portion of IgG1 or IgG3 to overcome the short half life of interleukin 2 and its systemic toxicity. See, Harvill et al., Immunotechnology, 1: 95-105 (1995).

In spite of the availability of TPO and MGDF, there remains a need to provide additional compounds that have a biological activity of stimulating the production of platelets (thrombopoietic activity) and/or platelet precursor cells, especially megakaryocytes (megakaryopoietic activity). The present invention provides new compounds having such activity(ies), and related aspects.

SUMMARY OF THE INVENTION

The present invention provides a group of compounds that are capable of binding to and triggering a transmembrane signal through, i.e., activating, the c-Mpl receptor, which is the same receptor that mediates the activity of endogenous thrombopoietin (TPO). Thus, the inventive compounds have thrombopoietic activity, i.e., the ability to stimulate, in vivo and in vitro, the production of platelets, and/or megakaryocytopoietic activity, i.e., the ability to stimulate, in vivo and in vitro, the production of platelet precursors.

In a first preferred embodiment, the inventive compounds comprise the following general structure:

$$TMP_1\text{-}(L_1)_n\text{-}TMP_2$$

wherein TMP_1 and TMP_2 are each independently selected from the group of compounds comprising the core structure: X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10},

wherein,

X2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X3 is selected from the group consisting of Gly and Ala;

X4 is Pro;

X5 is selected from the group consisting of Thr and Ser;

X6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X7 is selected from the group consisting of Arg and Lys;

X8 is selected from the group consisting of Gln, Asn, and Glu;

X9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X10 is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

L1 is a linker as described herein; and n is 0 or 1;

and physiologically acceptable salts thereof.

In one embodiment, L_1 comprises $(Gly)_n$, wherein n is 1 through 20, and when n is greater than 1, up to half of the Gly residues may be substituted by another amino acid selected from the remaining 19 natural amino acids or a stereoisomer thereof.

In addition to the core structure X_2-X_{10} set forth above for TMP_1 and TMP_2, other related structures are also possible wherein one or more of the following is added to the TMP_1 and/or TMP_2 core structure: X_1 is attached to the N-terminus and/or X_{11}, X_{12}, X_{13}, and/or X_{14} are attached to the C-terminus, wherein X_1, X_{12}, X_{13}, and X_{14} are as follows:

X_1 is selected from the group consisting of Ile, Ala, Val, Leu, Ser, and Arg;

X_{11}, is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Ser, Thr, Lys, His, and Glu;

X_{12} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Gly, Ser, and Gln;

X_{13} is selected from the group consisting of Arg, Lys, Thr, Val, Asn, Gln, and Gly; and

X_{14} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Thr, Arg, Glu, and Gly.

In a second preferred embodiment, the inventive compounds have the general formula:

$$(Fc)_m\text{-}(L_2)_q\text{-}TMP_1\text{-}(L_1)_n\text{-}TMP_2\text{-}(L_3)_r\text{-}(Fc)_p$$

wherein TMP_1, TMP_2 and n are each as described above; L_1, L_2 and L_3 are linker groups which are each independently selected from the linker groups described herein; Fc is an Fc region of an immunoglobulin (as defined herein below); m, p, q and r are each independently selected from the group consisting of 0 and 1, wherein at least one of m or p is 1, and further wherein if m is 0 then q is 0, and if p is 0, then r is 0; and physiologically acceptable salts thereof. In one embodiment, L_1, L_2, and L_3 independently comprise $(Gly)_n$, wherein n is 1 through 20, and when n is greater than 1, up to half of the Gly residues may be substituted by another amino acid selected from the remaining 19 natural amino acids or a stereoisomer thereof.

Derivatives of the above compounds (described below) are also encompassed by this invention.

The compounds of this invention are preferably peptides, and they may be prepared by standard synthetic methods or any other methods of preparing peptides.

The compounds of this invention that encompass non-peptide portions may be synthesized by standard organic chemistry reactions, in addition to standard peptide chemistry reactions when applicable.

The compounds of this invention may be used for therapeutic or prophylactic purposes by incorporating them with appropriate pharmaceutical carrier materials and administering an effective amount to a subject, such as a human (or other mammal). Other related aspects are also included in the instant invention.

BRIEF DESCRIPTION OF THE FIGURES

Numerous other aspects and advantages of the present invention will therefore be apparent upon consideration of the following detailed description thereof, reference being made to the drawings wherein:

FIG. **1** shows exemplary Fc polynucleotide and protein sequences (SEQ ID NO: 3 is the coding strand reading 5'-3', SEQ ID NO: 4 is the complementary strand reading 3'-5'; and SEQ ID NO: 5 is the encoded amino acids sequence) of human IgG1 that may be used in the Fc fusion compounds of this invention.

FIG. **2** shows a synthetic scheme for the preparation of pegylated peptide 19 (SEQ ID NO:17).

FIG. **3** shows a synthetic scheme for the preparation of pegylated peptide 20 (SEQ ID NO:18).

FIG. **4** shows the number of platelets generated in vivo in normal female BDF1 mice treated with one 100 μg/kg bolus injection of various compounds, as follows: PEG-MGDF means 20 kD average molecular weight PEG attached to the N-terminal amino group by reductive amination of amino acids 1-163 of native human TPO, which is expressed in *E. coli* (so that it is not glycosylated) (See WO 95/26746 entitled "Compositions and Methods for Stimulating Megakaryocyte Growth and Differentiation"); TMP means the compound of SEQ ID NO: 1; TMP-TMP means the compound of SEQ ID NO: 21; PEG-TMP-TMP means the compound of SEQ ID NO: 18, wherein the PEG group is a 5 kD average molecular weight PEG attached as shown in FIG. **3**; TMP-TMP-Fc is defined herein below and Fc-TMP-TMP is the same as TMP-TMP-Fc except that the Fc group is attached at the N-terminal end rather than the C-terminal end of the TMP-TMP peptide.

FIG. **5** shows the number of platelets generated in vivo in normal BDF1 mice treated with various compounds delivered via implanted osmotic pumps over a 7-day period. The compounds are defined in the same manner as set forth above for FIG. **4**.

FIGS. **6**A, **6**B, and **6**C show schematic diagrams of preferred compounds of the present invention. FIG. **6**A shows an Fc fusion compound wherein the Fc moiety is fused at the N-terminus of the TMP dimer, and wherein the Fc portion is a monomeric (single chain) form. FIG. **6**B shows an Fc fusion compound wherein the Fc region is fused at the N-terminus of the TMP dimer, and wherein the Fc portion is dimeric, and one Fc monomer is attached to a TMP dimer. FIG. **6**C shows an Fc fusion compound wherein the Fc moiety is fused at the N-terminus of the TMP dimer, and wherein the Fc portion is dimeric and each Fc monomer is attached to a TMP dimer.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In an effort to seek small structures as lead compounds in the development of therapeutic agents with more desirable properties, a different type of dimer of TMP and related structures were designed in which the C-terminus of one TMP peptide was linked to the N-terminus of a second TMP peptide, either directly or via a linker and the effects of this dimerization strategy on the bioactivity of the resulting dimeric molecules were then investigated. In some cases, these so-called tandem dimers (C-N link) were designed to have linkers between the two monomers, the linkers being preferably composed of natural amino acids, therefore rendering their synthesis accessible to recombinant technologies.

The present invention is based on the discovery of a group of compounds that have thrombopoietic activity and which are thought to exert their activity by binding to the endogenous TPO receptor, c-Mpl.

Compounds and Derivatives

In a first preferred embodiment, the inventive compounds comprise the following general structure:

$$TMP_1\text{-}(L_1)_n\text{-}TMP_2$$

wherein TMP_1 and TMP_2 are each independently selected from the group of compounds comprising the core structure: X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10},

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Trp, Tyr, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

L_1 is a linker as described herein; and

n is 0 or 1;

and physiologically acceptable salts thereof.

In one embodiment, L_1, comprises $(Gly)_n$, wherein n is 1 through 20, and when n is greater than 1, up to half of the Gly residues may be substituted by another amino acid selected from the remaining 19 natural amino acids or a stereoisomer thereof.

In addition to the core structure X_2-X_{10} set forth above for TMP_1 and TMP_2, other related structures are also possible wherein one or more of the following is added to the TMP_1 and/or TMP_2 core structure: X_1 is attached to the N-terminus and/or X_{11} X_{12} X_{13}, and/or X_{14} are attached to the C-terminus, wherein X_1, X_{11}, X_{12}, X_{13}, and X_{14} are as follows:

X_1 is selected from the group consisting of Ile, Ala, Val, Leu, Ser, and Arg;

X_{11} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Ser, Thr, Lys, His, and Glu;

X_{12} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Gly, Ser, and Gln;

X_{13} is selected from the group consisting of Arg, Lys, Thr, Val, Asn, Gln, and Gly; and

X_{14} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Thr, Arg, Glu, and Gly.

The term "TMP" is used to mean a moiety made up of, i.e., comprising, at least 9 subunits (X_2-X_{10}), wherein X_2-X_{10} comprise the core structure. The X_2-X_{14} subunits are preferably amino acids independently selected from among the 20 naturally-occurring amino acids, however, the invention embraces compounds where X_2-X_{14} are independently selected from the group of atypical, non-naturally occurring amino acids well known in the art. Specific preferred amino acids are identified for each position. For example, X_2 may be Glu, Asp, Lys, or Val. Both three-letter and single letter abbreviations for amino acids are used herein; in each case, the abbreviations are the standard ones used for the 20 naturally-occurring amino acids or well-known variations thereof. These amino acids may have either L or D stereochemistry (except for Gly, which is neither L nor D), and the TMPs may comprise a combination of stereochemistries. However, the L stereochemistry is preferred for all of the amino acids in the TMP chain. The invention also provides reverse TMP molecules wherein the amino terminal to carboxy terminal sequence of the amino acids is reversed. For example, the reverse of a molecule having the normal sequence X_1-X_2-X_3 would be X_3-X_2-X_1. The invention also provides retro-reverse TMP molecules wherein, like a reverse TMP, the amino terminal to carboxy terminal sequence of amino acids is reversed and residues that are normally "L" enatiomers in TMP are altered to the "D" stereoisomer form.

Additionally, physiologically acceptable salts of the TMPs are also encompassed. "Physiologically acceptable salts" means any salts that are known or later discovered to be pharmaceutically acceptable. Some specific preferred examples are: acetate, trifluoroacetate, hydrochloride, hydrobromide, sulfate, citrate, tartrate, glycolate, oxalate.

It is also contemplated that "derivatives" of the TMPs may be substituted for the above-described TMPs. Such derivative TMPs include moieties wherein one or more of the following modifications have been made:

one or more of the peptidyl [—C(O)NR—] linkages (bonds) have been replaced by a non-peptidyl linkage such as a —CH_2-carbamate linkage [—CH_2—OC(O)NR—]; a phosphonate linkage; a —CH_2-sulfonamide [—CH_2—$S(O)_2$NR—] linkage; a urea [—NHC(O)NH—] linkage; a —CH_2-secondary amine linkage; or an alkylated peptidyl linkage [—C(O)NR^6— where R^6 is lower alkyl];

peptides wherein the N-terminus is derivatized to a —NRR^1 group; to a —NRC(O)R group; to a —NRC(O)OR group; to a —$NRS(O)_2R$ group; to a —NHC(O)NHR group, where R and R^1 are hydrogen or lower alkyl, with the proviso that R and R^1 are not both hydrogen; to a succinimide group; to a benzyloxycarbonyl-NH— (CBZ-NH—) group; or to a benzyloxycarbonyl-NH— group having from 1 to 3 substituents on the phenyl ring selected from the group consisting of lower alkyl, lower alkoxy, chloro, and bromo; and

peptides wherein the free C terminus is derivatized to —$C(O)R^2$ where R^2 is selected from the group consisting of lower alkoxy and —NR^3R^4 where R^3 and R^4 are independently selected from the group consisting of hydrogen and lower alkyl. By "lower" is meant a group having from 1 to 6-carbon atoms.

Additionally, modifications of individual amino acids may be introduced into the TMP molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following are exemplary:

Lysinyl and amino terminal residues may be reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group.

The specific modification of tyrosyl residues per se has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R'—N=C=N—R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful for cross-linking the peptides or their functional derivatives to a water-insoluble support matrix of to other macromolecular carriers. Commonly used cross-linking agents include, e.g., 1,1-bis (diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 may be employed for protein immobilization.

Other possible modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, oxidation of the sulfur atom in Cys, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (Creighton, T. E., Proteins: Structure and Molecule Properties, W. H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N -terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Such derivatized moieties preferably improve one or more characteristics including thrombopoietic activity, solubility, absorption, biological half life, and the like of the inventive compounds. Alternatively, derivatized moieties result in compounds that have the same, or essentially the same, characteristics and/or properties of the compound that is not derivatized. The moieties may alternatively eliminate or attenuate any undesirable side effect of the compounds and the like.

In addition to the core structure set forth above, X_2-X_{10}, other structures that are specifically contemplated are those in which one or more additional X groups are attached to the core structure. Thus, X_1, and/or X_{11}, X_{12}, X_{13}, and X_{14} may be attached to the core structure. Some exemplary additional structures are the following:

X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14},

wherein X_1 through X_{14} are as described above. Each of TMP_1 and TMP_2 may be the same or different in sequence and/or length. In some preferred embodiments, TMP_1 and TMP_2 are the same.

A particularly preferred TMP is the following:

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Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp- (SEQ ID NO: 1)
Leu-Ala-Ala-Arg-Ala.
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As used herein "comprising" means, inter alia, that a compound may include additional amino acids on either or both of the - or C-termini of the given sequence. However, as long as a structure such as X_2 to X_{10}, X_1 to X_{14}, or one of the other exemplary structures is present, the remaining chemical structure is relatively less important. Of course, any structure outside of the core X_2 to X_{10} structure, or the X_1 to X_{14}, structure, should not significantly interfere with thrombopoietic activity of the compound. For example, an N-terminal Met residue is envisioned as falling within this invention. Additionally, although many of the preferred compounds of the invention are tandem dimers in that they possess two TMP moieties, other compounds of this invention are tandem multimers of the TMPs, i.e., compounds of the following exemplary structures:

TMP_1-L-TMP_2-L-TMP_3;
TMP_1-L-TMP_2-L-TMP_3-L-TMP_4;
TMP_1-L-TMP_2-L-TMP_3-L-TMP_4-L-TMP_5;

wherein TMP_1, TMP_2, TMP_3, TMP_4, and TMP_5 can have the same or different structures, and wherein each TMP and L is defined as set forth herein, and the linkers are each optional. Preferably, the compounds of this invention will have from 2-5 TMP moieties, particularly preferably 2-3, and most preferably 2. The compounds of the first embodiment of this invention will preferably have less than about 60, more preferably less than about 40 amino acids in total (i.e., they will be peptides).

As noted above, the compounds of the first embodiment of this invention are preferably TMP dimers which are either bonded directly or are linked by a linker group. The monomeric TMP moieties are shown in the conventional orientation from N to C terminus reading from left to right. Accordingly, it can be seen that the inventive compounds are all oriented so that the C terminus of TMP_1 is attached either directly or through a linker to the N-terminus of TMP_2. This orientation is referred to as a tandem orientation, and the inventive compounds may be generally referred to as "tandem dimers". These compounds are referred to as dimers even if TMP_1 and TMP_2 are structurally distinct. That is, both homodimers and heterodimers are envisioned.

The "linker" group ("L_1") is optional. When it is present, it is not critical what its chemical structure is, since it serves primarily as a spacer. The linker should be chosen so as not to interfere with the biological activity of the final compound and also so that immunogenicity of the final compound is not significantly increased. The linker is preferably made up of amino acids linked together by peptide bonds. Thus, in preferred embodiments, the linker comprises Y_n, wherein Y is a naturally occurring amino acid or a steroisomer thereof and "n" is any one of 1 through 20. The linker is therefore made up of from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally-occurring amino acids. In a more preferred embodiment, the 1 to 20 amino acids are selected from Gly, Ala, Pro, Asn, Gln, Cys, Lys. Even more preferably, the linker is made up of a majority of amino acids that are sterically un-hindered, such as Gly, Gly-Gly [$(Gly)_2$], Gly-Gly-Gly [$(Gly)_3$] . . . $(Gly)_{20}$, Ala, Gly-Ala, Ala-Gly, Ala-Ala, etc. Other specific examples of linkers are:

$(Gly)_3Lys(Gly)_4$; (SEQ ID NO: 6)

$(Gly)_3AsnGlySer(Gly)_2$ (SEQ ID NO: 7)

(this structure provides a site for glycosylation, when it is produced recombinantly in a mammalian cell system that is capable of glycosylating such sites);

$(Gly)_3Cys(Gly)_4$; (SEQ ID NO: 8) and

GlyProAsnGly. (SEQ ID NO: 9)

To explain the above nomenclature, for example, $(Gly)_3Lys(Gly)_4$ means Gly-Gly-Gly-Lys-Gly-Gly-Gly-Gly. Combinations of Gly and Ala are also preferred.

Non-peptide linkers are also possible. For example, alkyl linkers such as —HN—$(CH_2)_s$—CO—, wherein s=2-20 could be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6), lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, etc.

Another type of non-peptide linker is a polyethylene glycol group, such as:

—HN—CH_2—CH_2—(O—CH_2—CH_2)$_n$—O—CH_2—CO—

wherein n is such that the overall molecular weight of the linker ranges from approximately 101 to 5000, preferably 101 to 500.

In general, it has been discovered that a linker of a length of about 0-14 sub -units (e.g., amino acids) is preferred for the thrombopoietic compounds of the first embodiment of this invention.

The peptide linkers may be altered to form derivatives in the same manner as described above for the TMPs.

The compounds of this first group may further be linear or cyclic. By "cyclic" is meant that at least two separated, i.e., non-contiguous, portions of the molecule are linked to each other. For example, the amino and carboxy terminus of the ends of the molecule could be covalently linked to form a cyclic molecule. Alternatively, the molecule could contain two or more Cys residues (e.g., in the linker), which could cyclize via disulfide bond formation. It is further contemplated that more than one tandem peptide dimer can link to form a dimer of dimers. Thus, for example, a tandem dimer containing a Cys residue can form an intermolecular disulfide bond with a Cys of another such dimer. See, for example, the compound of SEQ ID NO: 20, below.

The compounds of the invention may also be covalently or noncovalently associated with a carrier molecule, such as a linear polymer (e.g., polyethylene glycol, polylysine, dextran, etc.), a branched-chain polymer (see, for example, U.S. Pat. No. 4,289,872 to Denkenwalter et al., issued Sep. 15, 1981; U.S. Pat. No. 5,229,490 to Tam, issued Jul. 20, 1993; WO 93/21259 by Frechet et al., published 28 Oct. 1993); a lipid; a cholesterol group (such as a steroid); or a carbohydrate or oligosaccharide. Other possible carriers include one or more water soluble polymer attachments such as polyoxyethylene glycol, or polypropylene glycol as described U.S. Pat. Nos. 4,640,835, 4,496,689, 4,301,144, 4,670,417, 4,791,192 and 4,179,337. Still other useful polymers known in the art include monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone)-polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol, as well as mixtures of these polymers.

A preferred such carrier is polyethylene glycol (PEG). The PEG group may be of any convenient molecular weight and may be straight chain or branched. The average molecular weight of the PEG will preferably range from about 2 kDa to about 100 kDa, more preferably from about 5 kDa to about 50 kDa, most preferably from about 5 kDa to about 10 kDa.

The PEG groups will generally be attached to the compounds of the invention via acylation, reductive alkylation, Michael addition, thiol alkylation or other chemoselective conjugation/ligation methods through a reactive group on the PEG moiety (e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group) to a reactive group on the target compound (e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group).

Carbohydrate (oligosaccharide) groups may conveniently be attached to sites that are known to be glycosylation sites in proteins. Generally, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids not counting proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneurarminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycosylated compound. Such site(s) may be incorporated in the linker of the compounds of this invention and are preferably glycosylated by a cell during recombinant production of the polypeptide compounds (e.g., in mammalian cells such as CHO, BHK, COS). However, such sites may further be glycosylated by synthetic or semi-synthetic procedures known in the art.

Some exemplary peptides of this invention are shown below. Single letter amino acid abbreviations are used, and the linker is shown separated by dashes for clarity. Additional abbreviations: BrAc means bromoacetyl ($BrCH_2C(O)$) and PEG is polyethylene glycol.

IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA	(SEQ ID NO: 10)
IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (cyclic)	(SEQ ID NO: 11)
IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (linear)	(SEQ ID NO: 12)
IEGPTLRQALAARA-GGGGGGGG-IEGPTLRQALAARA	(SEQ ID NO: 13)
IEGPTLRQWLAARA-GGGKGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 14)
IEGPTLRQWLAARA-GGGK(BrAc)GGGG-IEGPTLRQWLAARA	(SEQ ID NO: 15)
IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 16)
IEGPTLRQWLAARA-GGGK(PEG)GGGG-IEGPTLRQWLAARA	(SEQ ID NO: 17)
IEGPTLRQWLAARA-GGGC(PEG)GGGG-IEGPTLRQWLAARA	(SEQ ID NO: 18)
IEGPTLRQWLAARA-GGGNGSGG-IEGPTLRQWLAARA	(SEQ ID NO: 19)
IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 20)
IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA	
IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 21)

In each of the above compounds, an N-terminal Met (or M residue, using the one-letter code) is contemplated as well. Multimers (e.g., tandem and non-tandem, covalently bonded and non-covalently bonded) of the above compounds are also contemplated.

In a second embodiment of this invention, the compounds described above may further be fused to one or more Fc groups, either directly or through linker groups. In general, the formula of this second group of compounds is:

$$(Fc)_m\text{-}(L_2)_q\text{-}TMP_1\text{-}(L_1)_n\text{-}TMP_2\text{-}(L_3)_r\text{-}(Fc)_p$$

wherein TMP_1, TMP_2 and n are each as described above; L_1, L_2 and L_3 are linker groups which are each independently selected from the linker groups described above; Fc is an Fc region of an immunoglobulin; m, p, q and r are each independently selected from the group consisting of 0 and 1, wherein at least one of m or p is 1, and further wherein if m is 0 then q is 0, and if p is 0, then r is 0; and physiologically acceptable salts thereof.

Accordingly, the compounds of this second group have structures as defined for the first group of compounds as described above, but these compounds are further fused to at least one Fc group either directly or through one or more linker groups.

The Fc sequence of the above compounds may be selected from the human immunoglobulin IgG-1 heavy chain, see Ellison, J. W. et al., Nucleic Acids Res. 10:4071-4079 (1982), or any other Fc sequence known in the art (e.g. other IgG classes including but not limited to IgG-2, IgG-3 and IgG-4, or other immunoglobulins).

It is well known that Fc regions of antibodies are made up of monomeric polypeptide segments that may be linked into dimeric or multimeric forms by disulfide bonds or by non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on the class (e.g., IgG, IgA, IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, IgGA2) of antibody involved. The term "Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms of Fc molecules. It should be noted that Fc monomers will spontaneously dimerize when the appropriate Cys residues are present unless particular conditions are present that prevent dimerization through disulfide bond formation. Even if the Cys residues that normally form disulfide bonds in the Fc dimer are removed or replaced by other residues, the monomeric chains will generally dimerize through non-covalent interactions. The term "Fc" herein is used to mean any of these forms: the native monomer, the native dimer (disulfide bond linked), modified dimers (disulfide and/or non-covalently linked), and modified monomers (i.e., derivatives).

Variants, analogs or derivatives of the Fc portion may be constructed by, for example, making various substitutions of residues or sequences.

Variant (or analog) polypeptides include insertion variants, wherein one or more amino acid residues supplement an Fc amino acid sequence. Insertions may be located at either or both termini of the protein, or may be positioned within internal regions of the Fc amino acid sequence. Insertional variants with additional residues at either or both termini can include for example, fusion proteins and proteins including amino acid tags or labels. For example, the Fc molecule may optionally contain an N-terminal Met, especially when the molecule is expressed recombinantly in a bacterial cell such as *E. coli*.

In Fc deletion variants, one or more amino acid residues in an Fc polypeptide are removed. Deletions can be effected at one or both termini of the Fc polypeptide, or with removal of one or more residues within the Fc amino acid sequence. Deletion variants, therefore, include all fragments of an Fc polypeptide sequence.

In Fc substitution variants, one or more amino acid residues of an Fc polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature, however, the invention embraces substitutions that ore also non-conservative.

For example, cysteine residues can be deleted or replaced with other amino acids to prevent formation of some or all disulfide crosslinks of the Fc sequences. In particular, the amino acids at positions 7 and 10 of SEQ ID NO:5 are cysteine residues. One may remove each of these cysteine residues or substitute one or more such cysteine residues with other amino acids, such as Ala or Ser. As another example, modifications may also be made to introduce amino acid substitutions to (1) ablate the Fc receptor binding site; (2) ablate the complement (C1q) binding site; and/or to (3) ablate the antibody dependent cell-mediated cytotoxicity (ADCC) site. Such sites are known in the art, and any known substitutions are within the scope of Fc as used herein. For example, see Molecular Immunology, Vol. 29, No. 5, 633-639 (1992) with regards to ADCC sites in IgG1.

Likewise, one or more tyrosine residues can be replaced by phenylalanine residues as well. In addition, other variant amino acid insertions, deletions (e.g., from 1-25 amino acids) and/or substitutions are also contemplated and are within the scope of the present invention. Conservative amino acid substitutions will generally be preferred. Furthermore, alterations may be in the form of altered amino acids, such as peptidomimetics or D-amino acids.

Fc sequences of the TMP compound may also be derivatized, i.e., bearing modifications other than insertion, deletion, or substitution of amino acid residues. Preferably, the modifications are covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic, and inorganic moieties. Derivatives of the invention may be prepared to increase circulating half-life, or may be designed to improve targeting capacity for the polypeptide to desired cells, tissues, or organs.

It is also possible to use the salvage receptor binding domain of the intact Fc molecule as the Fc part of the inventive compounds, such as described in WO 96/32478, entitled "Altered Polypeptides with Increased Half-Life". Additional members of the class of molecules designated as Fc herein are those that are described in WO 97/34631, entitled "Immunoglobulin-Like Domains with Increased Half-Lives". Both of the published PCT applications cited in this paragraph are hereby incorporated by reference.

The Fc fusions may be at the N or C terminus of TMP_1 or TMP_2 or at both the N and C termini of the TMPs. It has been surprisingly discovered that peptides in which an Fc moiety is ligated to the N terminus of the TMP group is more bioactive than the other possibilities, so the fusion having an Fc domain at the N terminus of TMP_1 (i.e., r and p are both 0 and m and q are both 1 in general formula) is preferred. When the Fc chain is fused at the N-terminus of the TMP or linker, such fusion will generally occur at the C-terminus of the Fc chain, and vice versa.

Also preferred are compounds that are dimers (e.g., tandem and non-tandem) of the compounds set forth in the general formula as set out above. In such cases, each Fc chain will be linked to a tandem dimer of TMP peptides. A schematic example of such a compound is shown in FIG. **6**C. A preferred example of this type of compound is based on FIG. **6**C, wherein Fc is a dimer of the compound of SEQ ID NO: 5, each L_2 is $(Gly)_5$, TMP_1 and TMP_2 are each the compound of SEQ ID NO: 1, and each L_1 is $(Gly)_g$. This compound is also referred to herein as "Fc-TMP_1-L-TMP_2". It is also represented as a dimer (through the Fc portion) of SEQ ID NO: 34. The analogous compound wherein the Fc group is attached through a linker to the C-terminus of the TMP_2 groups in FIG. **6**C is also contemplated and is referred to herein as TMP_1-L-TMP_2-Fc.

Some specific examples of compounds from the second group are provided as follows:

Fc-IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA	(SEQ ID NO: 22)
Fc-IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA-Fc	(SEQ ID NO: 23)
IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA-Fc	(SEQ ID NO: 24)
Fc-GG-IEGPTLRQWLAA-GPNG-IEGPTLRQWLAARA	(SEQ ID NO: 25)
Fc-IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 26)
Fc-IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (cyclic)	(SEQ ID NO: 27)
Fc-IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (linear)	(SEQ ID NO: 28)
Fc-IEGPTLRQALAARA-GGGGGGGG-IEGPTLRQALAARA	(SEQ ID NO: 29)
Fc-IEGPTLRQWLAARA-GGGKGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 30)
Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 31)
Fc-IEGPTLRQWLAARA-GGGNGSGG-IEGPTLRQWLAARA	(SEQ ID NO: 32)
Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 33)
Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA	
Fc-GGGGG-IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA	(SEQ ID NO: 34)

In each of the above compounds, an additional N-terminal Met (or M residue, using the one-letter code) is contemplated as well. The Met residue may be attached at the N-terminus of the Fc group in those cases wherein there is an Fc group attached to the N-terminus of the TMP. In those cases wherein the Fc group is attached at the C-terminus of the TMP, the Met residues could be attached to the N-terminus of the TMP group.

In each of the above cases Fc is preferably the Fc region of the human immunoglobulin IgG1 heavy chain or a biologically active fragment, derivative, or dimer thereof, see Ellison, J. W. et al., Nucleic Acids Res. 10:4071-4079 (1982). The Fc sequence shown in SEQ ID NO: 5 is the most preferred Fc for the above compounds. Also preferred are the above compounds in which the Fc is a dimeric form of the sequence of SEQ ID NO: 5 and each Fc chain is attached to a TMP tandem dimer.

Additionally, although many of the preferred compounds of the second embodiment include one or more tandem dimers in that they possess two linked TMP moieties, other compounds of this invention include tandem multimers of the TMPs, i.e., compounds of the following exemplary structures:

Fc-TMP_1-L-TMP_2-L-TMP_3,

Fc-TMP_1-L-TMP_2-L-TMP_3-L-TMP_4;

Fc-TMP_1-L-TMP_2-L-TMP_3-L-TMP_4-L-TMP_5;

TMP_1-L-TMP_2-L-TMP_3-L-Fc;

TMP_1-L-TMP_2-L-TMP_3-L-TMP_4-L-Fc;

TMP_1-L-TMP_2-L-TMP_3-L-TMP_4-L-TMP_{-5}-L-Fc;

wherein TMP_1, TMP_2, TMP_3, TMP_4, and TMP_5 can have the same or different structures, and wherein Fc and each TMP and L is defined as set forth above, and the linkers are each optional. In each case above, the Fc group can be monomeric or dimeric, and in cases where the Fc is dimeric, one or more TMP multimer can be attached to each Fc chains. Also contemplated are other examples wherein the TMP dimers or multimers are attached to both the N and C-termini of one or both Fc chains, including the case wherein TMP dimers or multimers are attached to all four termini of two Fc chains.

Preferably, the compounds of this second embodiment of the invention will have from about 200 to 400 amino acids in total (i.e., they will be polypeptides).

Methods of Making

The compounds of this invention may be made in a variety of ways. Since many of the compounds will be peptides, or will include a peptide, methods for synthesizing peptides are of particular relevance here. For example, solid phase synthesis techniques may be used. Suitable techniques are well known in the art, and include those described in Merrifield, in Chem. Polypeptides, pp. 335-61 (Katsoyannis and Panayotis eds. 1973); Merrifield, J. Am. Chem. Soc. 85:2149 (1963); Davis et al., Biochem. Intl. 10:394-414 (1985); Stewart and Young, Solid Phase Peptide Synthesis (1969); U.S. Pat. No. 3,941,763; Finn et al., The Proteins, 3rd ed., vol. 2, pp. 105-253 (1976); and Erickson et al., The Proteins, 3rd ed., vol. 2, pp. 257-527 (1976). Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides.

The peptides may also be made in transformed host cells using recombinant DNA techniques. To do so, a recombinant DNA molecule coding for the peptide is prepared. Methods of preparing such DNA and/or RNA molecules are well known in the art. For instance, sequences coding for the peptides could be excised from DNA using suitable restriction enzymes. The relevant sequences can be created using the polymerase chain reaction (PCR) with the inclusion of useful restriction sites for subsequent cloning. Alternatively, the DNA/RNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidite method. Also, a combination of these techniques could be used.

The invention also includes a vector encoding the peptides in an appropriate host. The vector comprises the DNA molecule that encodes the peptides operatively linked to appropriate expression control sequences. Methods of effecting this operative linking, either before or after the peptide-encoding DNA molecule is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation.

The resulting vector comprising the peptide-encoding DNA molecule is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These factors include, for example, compatibility with the chosen expression vector, toxicity to the host cell of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence.

Within these general guidelines, useful microbial hosts include bacteria (such as *E. coli*), yeast (such as *Saccharomyces* sp. and *Pichia pastoris*) and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

Next, the transformed host is cultured under conventional fermentation conditions so that the desired peptides are expressed. Such fermentation conditions are well known in the art.

Finally, the peptides are purified from the fermentation culture or from the host cells in which they are expressed. These purification methods are also well known in the art.

Compounds that contain derivatized peptides or which contain non-peptide groups may be synthesized by well-known organic chemistry techniques.

Uses of the Compounds

The compounds of this invention have the ability to bind to and activate the c-Mpl receptor, and/or have the ability to stimulate the production (both in vivo and in vitro) of platelets ("thrombopoietic activity") and platelet precursors ("megakaryocytopoietic activity"). To measure the activity (-ies) of these compounds, one can utilize standard assays, such as those described in WO95/26746 entitled "Compositions and Methods for Stimulating Megakaryocyte Growth and Differentiation". In vivo assays are further described in the Examples section herein.

The conditions to be treated by the methods and compositions of the present invention are generally those which involve an existing megakaryocyte/platelet deficiency or an expected or anticipated megakaryocyte/platelet deficiency in the future (e.g., because of planned surgery or platelet donation). Such conditions may be the result of a deficiency (temporary or permanent) of active Mpl ligand in vivo. The generic term for platelet deficiency is thrombocytopenia, and hence the methods and compositions of the present invention are generally available for prophylactically or therapeutically treating thrombocytopenia in patients in need thereof.

The World Health Organization has classified the degree of thrombocytopenia on the number of circulating platelets in the individual (Miller, et al., Cancer 47:210-211 (1981)). For example, an individual showing no signs of thrombocytopenia (Grade 0) will generally have at least 100,000 platelets/mm^3. Mild thrombocytopenia (Grade 1) indicates a circulating level of platelets between 79,000 and 99,000/mm^3. Moderate thrombocytopenia (Grade 2) shows between 50,000 and 74,000 platelets/mm^3 and severe thrombocytopenia is characterized by between 25,000 and 49,000 platelets/mm^3. Life-threatening or debilitating thrombocytopenia is characterized by a circulating concentration of platelets of less than 25,000/mm^3.

Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other specific disease conditions. Exemplary specific disease conditions that involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia; idiopathic or immune thrombocytopenia (ITP), including idiopathic thrombocytopenic purpura associated with breast cancer; HIV associated ITP and HIV-related thrombotic thrombocytopenic purpura; metastatic tumors which result in thrombocytopenia; systemic lupus erythematosus; including neonatal lupus syndrome splenomegaly; Fanconi's syndrome; vitamin B12 deficiency; folic acid deficiency; May-Hegglin anomaly; Wiskott-Aldrich syndrome; chronic liver disease; myelodysplastic syndrome associated with thrombocytopenia; paroxysmal nocturnal hemoglobinuria; acute profound thrombocytopenia following C7E3 Fab (Abciximab) therapy; alloimmune thrombocytopenia, including maternal alloimmune thrombocytopenia; thrombocytopenia associated with antiphospholipid antibodies and thrombosis; autoimmune thrombocytopenia; drug-induced immune thrombocytopenia, including carboplatin-induced thrombocytopenia, heparin-induced thrombocytopenia; fetal thrombocytopenia; gestational thrombocytopenia; Hughes' syndrome; lupoid thrombocytopenia; accidental and/or massive blood loss; myeloproliferative disorders; thrombocytopenia in patients with malignancies; thrombotic thrombocytopenia purpura, including thrombotic microangiopathy manifesting as thrombotic thrombocytopenic purpura/hemolytic uremic syndrome in cancer patients; autoimmune hemolytic anemia; occult jejunal diverticulum perforation; pure red cell aplasia; autoimmune thrombocytopenia; nephropathia epidemica; rifampicin-associated acute renal failure; Paris-Trousseau thrombocytopenia; neonatal alloimmune thrombocytopenia; paroxysmal nocturnal hemoglobinuria; hematologic changes in stomach cancer; hemolytic uremic syndromes in childhood; hematologic manifestations related to viral infection including hepatitis A virus and CMV-associated thrombocytopenia. Also, certain treatments for AIDS result in thrombocytopenia (e.g., AZT). Certain wound healing disorders might also benefit from an increase in platelet numbers.

With regard to anticipated platelet deficiencies, e.g., due to future surgery, a compound of the present invention could be administered several days to several hours prior to the need for platelets. With regard to acute situations, e.g., accidental and massive blood loss, a compound of this invention could be administered along with blood or purified platelets.

The compounds of this invention may also be useful in stimulating certain cell types other than megakaryocytes if such cells are found to express Mpl receptor. Conditions associated with such cells that express the Mpl receptor, which are responsive to stimulation by the Mpl ligand, are also within the scope of this invention.

The compounds of this invention may be used in any situation in which production of platelets or platelet precursor cells is desired, or in which stimulation of the c-Mpl receptor is desired. Thus, for example, the compounds of this invention may be used to treat any condition in a mammal wherein there is a need of platelets, megakaryocytes, and the like. Such conditions are described in detail in the following exemplary sources: WO95/26746; WO95/21919; WO95/18858; WO95/21920 and are incorporated herein.

The compounds of this invention may also be useful in maintaining the viability or storage life of platelets and/or megakaryocytes and related cells. Accordingly, it could be useful to include an effective amount of one or more such compounds in a composition containing such cells.

By "mammal" is meant any mammal, including humans, domestic animals including dogs and cats; exotic and/or zoo animals including monkeys; laboratory animals including mice, rats, and guinea pigs; farm animals including horses, cattle, sheep, goats, and pigs; and the like. The preferred mammal is human.

Pharmaceutical Compositions

The present invention also provides methods of using pharmaceutical compositions of the inventive compounds. Such pharmaceutical compositions may be for administration for injection, or for oral, nasal, transdermal or other forms of administration, including, e.g., by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, intraocular, retrobulbar, intrapulmonary (e.g., aerosolized drugs) or subcutaneous injection (including depot administration for long term release); by sublingual, anal, vaginal, or by surgical implantation, e.g., embedded under the splenic capsule, brain, or in the cornea. The treatment may consist of a single dose or a plurality of doses over a period of time. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of a compound of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. The pharmaceutical compositions optionally may include still other pharmaceutically acceptable liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media, including but are not limited to, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, starches, sucrose, dextrose, gum acacia, calcium phosphate, mineral oil, cocoa butter, and oil of *theobroma*. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.

Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton Pa. 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Pat. No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Pat. No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K., Modern Pharmaceutics, Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979, herein incorporated by reference. In general, the formulation will include the inventive compound, and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above inventive compounds. If necessary, the compounds may be chemically modified so that oral delivery is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the compound molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the compound and increase in circulation time in the body. Examples of such moieties include: Polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline (Abuchowski and Davis, Soluble Polymer-Enzyme Adducts, Enzymes as Drugs, Hocenberg and Roberts, eds., Wiley-Interscience, New York, N.Y., (1981), pp 367-383; Newmark, et al., J. Appl. Biochem. 4:185-189 (1982)). Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

For the oral delivery dosage forms, it is also possible to use a salt of a modified aliphatic amino acid, such as sodium N-(8-[2-hydroxybenzoyl]amino) caprylate (SNAC), as a carrier to enhance absorption of the therapeutic compounds of this invention. The clinical efficacy of a heparin formulation using SNAC has been demonstrated in a Phase II trial conducted by Emisphere Technologies. See U.S. Pat. No. 5,792,451, "Oral drug delivery composition and methods".

The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, α-lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment, a surfactant might be added as a wetting agent.

Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the compound are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulation may be desirable. The drug could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms e.g., gums. Slowly degenerating matrices may also be incorporated into the formulation, e.g., alginates, polysaccharides. Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e., the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film coated tablet and the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Also contemplated herein is pulmonary delivery of the present protein (or derivatives thereof). The protein (or derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream. (Other reports of this include Adjei et al., Pharmaceutical Research 7:565-569 (1990); Adjei et al., International Journal of Pharmaceutics 63:135-144 (1990)(leuprolide acetate); Braquet et al., Journal of Cardiovascular Pharmacology 13 (suppl.5): s.143-146 (1989)(endothelin-1); Hubbard et al., Annals of Internal Medicine 3:206-212 (1989)(α1-antitrypsin); Smith et al., J. Clin. Invest. 84:1145-1146 (1989) (α1-proteinase); Oswein et al., "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II, Keystone, Colo., March, 1990 (recombinant human growth hormone); Debs et al., The Journal of Immunology 140:3482-3488 (1988)(interferon-γ and tumor necrosis factor α) and Platz et al., U.S. Pat. No. 5,284,656 (granulocyte colony stimulating factor).

Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially available devices suitable for the practice of this invention are the ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Mo.; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colo.; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, N.C.; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Mass.

All such devices require the use of formulations suitable for the dispensing of the inventive compound. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to diluents, adjuvants and/or carriers useful in therapy.

The inventive compound should most advantageously be prepared in particulate form with an average particle size of less than 10 μm (or microns), most preferably 0.5 to 5 μm, for most effective delivery to the distal lung.

Carriers include carbohydrates such as trehalose, mannitol, xylitol, sucrose, lactose, and sorbitol. Other ingredients for use in formulations may include DPPC, DOPE, DSPC and DOPC. Natural or synthetic surfactants may be used. Polyethylene glycol may be used (even apart from its use in derivatizing the protein or analog). Dextrans, such as cyclodextran, may be used. Bile salts and other related enhancers may be used. Cellulose and cellulose derivatives may be used. Amino acids may be used, such as use in a buffer formulation.

Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the inventive compound dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per mL of solution. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the inventive compound suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing the inventive compound and may also include a bulking agent, such as lactose, sorbitol, sucrose, mannitol, trehalose, or xylitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation.

Nasal delivery of the inventive compound is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran. Delivery via transport across other mucous membranes is also contemplated.

Dosages

The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the dose should be in the range of 0.1 μg to 100 mg of the inventive compound per kilogram of body weight per day, preferably 0.1 to 1000 μg/kg; and more preferably 0.1 to 150 μg/kg, given in daily doses or in equivalent doses at longer or shorter intervals, e.g., every other day, twice weekly, weekly, or twice or three times daily.

The inventive compound may be administered by an initial bolus followed by a continuous infusion to maintain therapeutic circulating levels of drug product. As another example, the inventive compound may be administered as a one-time dose. Those of ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient. The frequency of dosing will depend on the pharmacokinetic parameters of the agents and the route of administration. The optimal pharmaceutical formulation will be determined by one skilled in the art depending upon the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such formulations may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein, as well as the pharmacokinetic data observed in the human clinical trials discussed above. Appropriate dosages may be ascertained through use of established assays for determining blood levels dosages in conjunction with appropriate dose-response data. The final dosage regimen will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the drug's specific activity, the severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels and duration of treatment for various diseases and conditions.

The therapeutic methods, compositions and compounds of the present invention may also be employed, alone or in combination with other cytokines, soluble Mpl receptor, hematopoietic factors, interleukins, growth factors or antibodies in the treatment of disease states characterized by other symptoms as well as platelet deficiencies. It is anticipated that the inventive compound will prove useful in treating some forms of thrombocytopenia in combination with general stimulators of hematopoiesis, such as IL-3 or GM-CSF. Other megakaryocytic stimulatory factors, i.e., meg-CSF, stem cell factor (SCF), leukemia inhibitory factor (LIF), oncostatin M (OSM), or other molecules with megakaryocyte stimulating activity may also be employed with Mpl ligand. Additional exemplary cytokines or hematopoietic factors for such co-administration include IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-i (CSF-1), M-CSF, SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, IFN-gamma, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, thrombopoietin (TPO), angiopoietins, for example Ang-1, Ang-2, Ang-4, Ang-Y, the human angiopoietin-like polypeptide, vascular endothelial growth factor (VEGF), angiogenin, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic proteins, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, brain derived neurotrophic factor, ciliary neutrophic factor, ciliary neutrophic factor receptor α, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2 α, cytokine-induced neutrophil chemotactic factor 2 β, β endothelial cell growth factor, endothelin 1, epidermal growth factor, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6, fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor 8b, fibroblast growth factor 8c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophic factor receptor α 1, glial cell line-derived neutrophic factor receptor α 2, growth related protein, growth related protein a, growth related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neurotrophin-3, neurotrophin-4, placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor receptor, TNF, including TNF0, TNF1, TNF2, transforming growth factor α, transforming growth factor β, transforming growth factor β1, transforming growth factor β1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β binding protein III, tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, vascular endothelial growth factor, and chimeric proteins and biologically or immunologically active fragments thereof. It may further be useful to administer, either simultaneously or sequentially, an effective amount of a soluble mammalian Mpl receptor, which appears to have an effect of causing megakaryocytes to fragment into platelets once the megakaryocytes have reached mature form. Thus, administration of an inventive compound (to enhance the number of mature megakaryocytes) followed by administration of the soluble Mpl receptor (to inactivate the ligand and allow the mature megakaryocytes to produce platelets) is expected to be a particularly effective means of stimulating platelet production. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

In cases where the inventive compounds are added to compositions of platelets and/or megakaryocytes and related cells, the amount to be included will generally be ascertained experimentally by techniques and assays known in the art. An exemplary range of amounts is 0.1 μg-1 mg inventive compound per 10^6 cells.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below.

EXAMPLES

I. The following sets forth exemplary methods for making some of the compounds of the first group disclosed herein.

A. Materials and Methods

All amino acid derivatives (all of L-configurations) and resins used in peptide synthesis were purchased from Novabiochem. Peptide synthesis reagents (DCC, HOBt, etc.) were purchased in the solution forms from Applied Biosystems, Inc. The two PEG derivatives were from Shearwater Polymers, Inc. All solvents (dichloromethane, N-methylpyrrolidinone, methanol, acetonitrile) were from EM Sciences. Analytical BPLC was run on a Beckman system with a Vydac column (0.46 cm×25 cm, C18 reversed phase, 5 mm), at a flow rate of 1 ml/min and with dual UV detection at 220 and 280 nm. Linear gradients were used for all HPLC operations with two mobile phases: Buffer A—H_2O (0.1% TFA) and Buffer B—acetonitrile (0.1% TFA). The numbered peptides referred to herein, e.g., 17b, 18, 19, and 20, are numbered in reference to Table 1, and some of them are further illustrated in FIGS. **2** and **3**.

Peptide synthesis. All peptides were prepared by the well established stepwise solid phase synthesis method. Solid-phase synthesis with Fmoc chemistry was carried out using an ABI Peptide Synthesizer. Typically, peptide synthesis began with a preloaded Wang resin on a 0.1 mmol scale. Fmoc deprotection was carried out with the standard piperidine protocol. The coupling was effected using DCC/HOBt. Side-chain protecting groups were: Glu(O-t-Bu), Thr(t-Bu), Arg (Pbf), Gln(Trt), Trp(t-Boc) and Cys(Trt). For the first peptide precursor for pegylation, Dde was used for side chain protection of the Lys on the linker and Boc-Ile-OH was used for the last coupling. Dde was removed by using anhydrous hydrazine (2% in NMP, 3×2 min), followed by coupling with bromoacetic anhydride preformed by the action of DCC. For peptide 18, the cysteine side chain in the linker was protected by a trityl group. The final deprotection and cleavage of all peptidyl-resins was effected at RT for 4 hr, using trifluoroacetic acid (TFA) containing 2.5% H_2O, 5% phenol, 2.5% triisopropylsilane and 2.5% thioanisole. After removal of TFA, the cleaved peptide was precipitated with cold anhydrous ether. Disulfide formation of the cyclic peptide was performed directly on the crude material by using 15% DMSO in H_2O (pH 7.5). All crude peptides were purified by preparative reverse phase HPLC and the structures were confirmed by ESI-MS and amino acid analysis.

Alternatively, all peptides described above could also be prepared by using the t-Boc chemistry. In this case, the starting resins would be the classic Merrifield or Pam resin, and side chain protecting groups would be: Glu(OBzl), Thr(Bzl), Arg(Tos), Trp(CHO), Cys(p-MeBzl). Hydrogen fluoride (HF) would be used for the final cleavage of the peptidyl resins.

All the tandem dimeric peptides described in this study that have linkers composed of natural amino acids can also be prepared by recombinant DNA technology.

PEGylation. A novel, convergent strategy for the pegylation of synthetic peptides was developed which consists of combining, through forming a conjugate linkage in solution, a peptide and a PEG moiety, each bearing a special functionality that is mutually reactive toward the other. The precursor peptides can be easily prepared with the conventional solid phase synthesis as described above. As described below, these peptides are "preactivated" with an appropriate functional group at a specific site. The precursors are purified and fully characterized prior to reacting with the PEG moiety. Ligation of the peptide with PEG usually takes place in aqueous phase and can be easily monitored by reverse phase analytical HPLC. The pegylated peptides can be easily purified by preparative HPLC and characterized by analytical HPLC, amino acid analysis and laser desorption mass spectrometry.

Preparation of peptide 19. Peptide 17b (12 mg) and MeO-PEG-SH 5000 (30 mg, 2 equiv.) were dissolved in 1 ml aqueous buffer (pH 8). The mixture was incubated at RT for about 30 min and the reaction was checked by analytical HPLC which showed a >80% completion of the reaction. The pegylated material was isolated by preparative HPLC.

Preparation of peptide 20. Peptide 18 (14 mg) and MeO-PEG-maleimide (25 mg) were dissolved in about 1.5 ml aqueous buffer (pH 8). The mixture was incubated at RT for about 30 min, at which time ~70% transformation was complete as monitored with analytical HPLC by applying an aliquot of sample to the HPLC column. The pegylated material was purified by preparative HPLC.

Bioactivity assay. The TPO in vitro bioassay is a mitogenic assay utilizing an IL-3 dependent clone of murine 32D cells that have been transfected with human mpl receptor. This assay is described in greater detail in WO 95/26746. Cells are maintained in MEM medium containing 10% Fetal Clone II and 1 ng/ml mIL-3. Prior to sample addition, cells are prepared by rinsing twice with growth medium lacking mIL-3. An extended twelve point TPO standard curve is prepared, ranging from 3333 to 39 pg/ml. Four dilutions, estimated to fall within the linear portion of the standard curve, (1000 to 125 pg/ml), are prepared for each sample and run in triplicate. A volume of 100 μl of each dilution of sample or standard is added to appropriate wells of a 96 well microtiter plate containing 10,000 cells/well. After forty-four hours at 37° C. and 10% CO_2, MTS (a tetrazolium compound which is bioreduced by cells to a formazan) is added to each well. Approximately six hours later, the optical density is read on a plate reader at 490 nm. A dose response curve (log TPO concentration vs. O.D.-Background) is generated and linear regression analysis of points which fall in the linear portion of the standard curve is performed. Concentrations of unknown test samples are determined using the resulting linear equation and a correction for the dilution factor.

Abbreviations. HPLC: high performance liquid chromatography; ESI-MS: Electron spray ionization mass spectrometry; MALDI-MS: Matrix-assisted laser desorption ionization mass spectrometry; PEG: Poly(ethylene glycol). All amino acids are represented in the standard three-letter or single-letter codes, t-Boc: tert-Butoxycarbonyl; tBu: tert-Butyl; Bzl: Benzyl; DCC: Dicylcohexylcarbodiimide; HOBt: 1-Hydroxybenzotriazole; NMP: N-methyl-2-pyrrolidinone; Pbf: 2,2,4,6,7-pendamethyldihydro-benzofuran-5-sulfonyl; Trt: trityl; Dde: 1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene) ethyl.

B. Results

TMP tandem dimers with polyglycine linkers. The design of sequentially linked TMP dimers was based on the assumption that a dimeric form of TMP was required for its effective interaction with c-Mpl (the TPO receptor) and that depending on how they were wound up against each other in the receptor context, the two TMP molecules could be tethered together in the C- to N-terminus configuration in a way that would not perturb the global dimeric conformation. Clearly, the activity of the tandem linked dimers may also depend on proper selection of the length and composition of the linker that joins the C- and -termini of the two sequentially aligned TMP monomers. Since no structural information of the TMP bound to c-Mpl was available, a series of dimeric peptides with linkers composed of 0 to 10 and 14 glycine residues (Table 1) were synthesized. Glycine was chosen because of its simplicity and flexibility. It was reasoned that a flexible polyglycine peptide chain might allow for the free folding of the two tethered TMP repeats into the required conformation, while more sterically hindered amino acid sequences may adopt undesired secondary structures whose rigidity might disrupt the correct packing of the dimeric peptide in the receptor context.

The resulting peptides are readily accessible by conventional solid phase peptide synthesis methods (Merrifiled, R. B., Journal of the American Chemical Society 85:2149

(1963)) with either Fmoc or t-Boc chemistry. Unlike the synthesis of the C-terminally linked parallel dimer (SEQ ID NO: 2) which required the use of an orthogonally protected lysine residue as the initial branch point to build the two peptide chains in a pseudosymmetrical way (Cwirla, S. E. et al., Science 276:1696-1699 (1997)), the synthesis of our tandem dimers was a straightforward, stepwise assembly of the continuous peptide chains from the C- to N-terminus. Since dimerization of TMP had a more dramatic effect on the proliferative activity than binding affinity as shown for the C-terminal dimer (Cwirla, S. E. et al., Science 276:1696-1699 (1997)), the synthetic peptides were tested directly for biological activity in a TPO-dependent cell-proliferation assay using an IL-3 dependent clone of murine 32D cells transfected with the full-length c-Mpl (Palacios, R. et al., Cell 41:727 (1985)). As the test results showed (see Table 1 below), all of the polyglycine linked tandem dimers demonstrated >1000 fold increases in potency as compared to the monomer, and were even more potent than the C-terminal dimer in this cell proliferation assay. The absolute activity of the C-terminal dimer in our assay was lower than that of the native TPO protein, which is different from the previously reported findings in which the C-terminal dimer was found to be as active as the natural ligand (Cwirla, S. E. et al., Science 276:1696-1699 (1997)). This might be due to differences in the conditions used in the two assays. Nevertheless, the difference in activity between tandem dimers © terminal of first monomer linked to N terminal of second monomer) and parallel dimers © terminal of first monomer linked to C terminal of second monomer) in the same assay clearly demonstrated the superiority of tandem dimerized product compared to parallel dimer products. It is interesting to note that a wide range of length is tolerated by the linker. The optimal linker with the selected TMP monomers (SEQ ID NO: 1) apparently is composed of 8 glycines.

Other tandem dimers. Subsequent to this first series of TMP tandem dimers, several other molecules were designed either with different linkers or containing modifications within the monomer itself. The first of these molecules, peptide 13, has a linker composed of GPNG, a sequence known to have a high propensity to form a β-turn-type secondary structure. Although still about 100-fold more potent than the monomer, this peptide was found to be >10-fold less active than the GGGG-linked analog. Thus, introduction of a relatively rigid β-turn at the linker region seemed to cause a slight distortion of the optimal agonist conformation in this short linker form.

The Trp9 in the TMP sequence is a highly conserved residue among the active peptides isolated from random peptide libraries. There is also a highly conserved Trp in the consensus sequences of EPO mimetic peptides and this Trp residue was found to be involved in the formation of a hydrophobic core between the two EPO Mimetic Peptides (EMPs) and contributed to hydrophobic interactions with the EPO receptor (Livnah, O. et al., Science 273:464471 (1996)). By analogy, it was thought that the Trp9 residue in TMP might have a similar function in dimerization of the peptide ligand, and in an attempt to modulate and estimate the effects of noncovalent hydrophobic forces exerted by the two indole rings, several analogs were constructed resulting from mutations at the Trp. So in peptide 14, the Trp residue in each of the two TMP monomers was replaced with a Cys, and an intramolecular disulfide bond was formed between the two cysteines by oxidation which was envisioned to mimic the hydrophobic interactions between the two Trp residues in peptide dimerization. Peptide 15 is the reduced form of peptide 14. In peptide 16, the two Trp residues were replaced by Ala. As the assay data show, all three analogs were inactive. These data further demonstrated that Trp is important for the activity of the TPO mimetic peptide, not just for dimer formation.

The next two peptides (peptide 17a, and 18) each contain in their 8-amino acid linker a Lys or Cys residue. These two compounds are precursors to the two pegylated peptides (peptide 19 and 20) in which the side chain of the Lys or Cys is modified by a polyethylene glycol (PEG) moiety. It was decided to introduce a PEG moiety in the middle of a relatively long linker, so that the large PEG component (5 kDa) is far enough away from the important binding sites in the peptide molecule. PEG is a known biocompatible polymer which is increasingly used as a covalent modifier to improve the pharmacokinetic profiles of peptide- and protein-based therapeutics.

A modular, solution based method was devised for convenient pegylation of synthetic or recombinant peptides. The method is based on the now well established chemoselective ligation strategy which utilizes the specific reaction between a pair of mutually reactive functionalities. So, for pegylated peptide 19, the lysine side chain was preactivated with a bromoacetyl group to give peptide 17b to accommodate reaction with a thiol-derivatized PEG. To do that, an orthogonal protecting group, Dde, was employed for the protection of the lysine E-amine. Once the whole peptide chain was assembled, the N-terminal amine was reprotected with t-Boc. Dde was then removed to allow for the bromoacetylation. This strategy gave a high quality crude peptide which was easily purified using conventional reverse phase HPLC. Ligation of the peptide with the thiol-modified PEG took place in aqueous buffer at pH 8 and the reaction completed within 30 min. MALDI-MS analysis of the purified, pegylated material revealed a characteristic, bell-shaped spectrum with an increment of 44 Da between the adjacent peaks. For PEG-peptide 20, a cysteine residue was placed in the linker region and its side chain thiol group would serve as an attachment site for a maleimide-containing PEG. Similar conditions were used for the pegylation of this peptide. As the assay data revealed, these two pegylated peptides had even higher in vitro bioactivity as compared to their unpegylated counterparts.

Peptide 21 has in its 8-amino acid linker a potential glycosylation motif, NGS. Since the exemplary tandem dimers are made up of natural amino acids linked by peptide bonds, expression of such a molecule in an appropriate eukaryotic cell system should produce a glycopeptide with the carbohydrate moiety added on the side chain carboxyamide of Asn. Glycosylation is a common post-translational modification process which can have many positive impacts on the biological activity of a given protein by increasing its aqueous solubility and in vivo stability. As the assay data show, incorporation of this glycosylation motif into the linker maintained high bioactivity. The synthetic precursor of the potential glycopeptide had in effect an activity comparable to that of the $-(Gly)_8-$ linked analog. Once glycosylated, this peptide is expected to have the same order of activity as the pegylated peptides, because of the similar chemophysical properties exhibited by a PEG and a carbohydrate moiety.

The last peptide is a dimer of a dimer. It was prepared by oxidizing peptide 18, which formed an intermolecular disulfide bond between the two cysteine residues located at the linker. This peptide was designed to address the possibility that TMP was active as a tetramer. The assay data showed that this peptide was not more active than an average tandem dimer on an adjusted molar basis, which indirectly supports the idea that the active form of TMP is indeed a dimer, otherwise dimerization of a tandem dimer would have a further impact on the bioactivity.

The following Table I summarizes relative activities of the above-described compounds in terms of the EC50 based on in vitro assays as described above.

TABLE I

Compound		Relative Potency
	TPO	4.0
TMP monomer (SEQ ID NO: 1)		1.0
TMP C—C dimer (SEQ ID NO: 2)		3.5
TMP-(Gly)$_n$-TMP:		
1	n = 0	4.5
2	n = 1	4.0
3	n = 2	4.0
4	n = 3	4.0
5	n = 4	4.0
6	n = 5	4.0
7	n = 6	4.0
8	n = 7	4.0
9	n = 8	4.5
10	n = 9	4.0
11	n = 10	4.0
12	n = 14	4.0
13	TMP-GPNG-TMP (SEQ ID NO. 10)	3.0
14	IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (SEQ ID NO. 11)	0.5
15	IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (SEQ ID NO. 12)	0.5
16	IEGPTLRQALAARA-GGGGGGGG-IEGPTLRQALAARA (SEQ ID NO. 13)	0.5
17a	TMP-GGGKGGGG-TMP (SEQ ID NO. 14)	4.0
17b	TMP-GGGK(BrAc)GGGG-TMP (SEQ ID NO. 15)	ND
18	TMP-GGGCGGGG-TMP (SEQ ID NO. 16)	4.0
19	TMP-GGGK(PEG)GGGG-TMP (SEQ ID NO. 17)	5.0
20	TMP-GGGC(PEG)GGGG-TMP (SEQ ID NO. 18)	5.0
21	TMP-GGGNGSGG-TMP (SEQ ID NO. 19)	4.0
22	TMP-GGGCGGGG-TMP (SEQ ID NO. 20) TMP-GGGCGGGG-TMP	4.0

NOTE:
In Table 1, numerals indicate approximately 1 log of activity, so that the difference in activity between "1" and "4" is approximately 1000-fold. An increment of 0.5 is an intermediate point, so that the difference in activity between "1" and "3.5" is approximately 500-fold. "ND" means not determined.

II. The following sets forth exemplary methods for making some of the compounds of the second group disclosed herein.

A. Preparation of an Fc Fusion Compound of the Type Shown in FIG. 6C.

A DNA sequence coding for the Fc region of human IgG1 fused in-frame to a dimer of the TPO-mimetic peptide (SEQ ID NO: 34) was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as follows.

The fusion gene was constructed using standard PCR technology. Templates for PCR reactions were the fusion vector containing the Fc sequence and a synthetic gene encoding the remainder of the compound of SEQ ID NO: 34. The synthetic gene was constructed from the 4 overlapping oligonucleotides shown below:

```
1830-52 AAA GGT GGA GGT GGT GGT ATC  (SEQ ID NO: 35)
        GAA GGT CCG ACT CTG CGT CAG
        TGG CTG GCT GCT CGT GCT
```

-continued

```
1830-53 ACC TCC ACC ACC AGC ACG AGC  (SEQ ID NO: 36)
        AGC CAG CCA CTG ACG CAG AGT
        CGG ACC

1830-54 GGT GGT GGA GGT GGC GGC GGA  (SEQ ID NO: 37)
        GGT ATT GAG GGC CCA ACC CTT
        CGC CAA TGG CTT GCA GCA CGC
        GCA

1830-55 AAA AAA AGG ATC CTC GAG ATT  (SEQ ID NO: 38)
        ATG CGC GTG CTG CAA GCC ATT
        GGC GAA GGG TTG GGC CCT CAA
        TAC CTC CGC CGC C
```

The 4 oligonucleotides were annealed to form the duplex shown below:

```
AAAGGTGGAGGTGGTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCT

1---------+---------+---------+---------+---------+---------+  60

                        CCAGGCTGAGACGCAGTCACCGACCGACGAGCACGA

 K  G  G  G  G  G  I  E  G  P  T  L  R  Q  W  L  A  A  R  A
```

-continued

```
GGTGGTGGAGGTGGCGGCGGAGGTATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCA
---------+---------+---------+---------+---------+---------+  120
CCACCACCTCCACCGCCGCCTCCATAACTCCCGGGTTGGGAAGCGGTTACCGAACGTCGT
 G  G  G  G  G  G  G  G  I  E  G  P  T  L  R  Q  W  L  A  A

CGCGCA
--------------------------                                    148
GCGCGTATTAGAGCTCCTAGGAAAAAAA
 R  A  *
```

SEQ ID NO: 39 [co-linear oligonucleotides 1830-52 and 1830-54]

SEQ ID NO: 40 [co-linear oligonucleotides 1830-53 and 1830-55]

and SEQ ID NO: 41 [the encoded amino acid sequence].

This duplex was amplified in a PCR reaction using 1830-52 and 1830-55 as the sense and antisense primers.

The Fc portion of the molecule was generated in a PCR reaction with Fc DNA using the primers

```
1216-52  AAC ATA AGT ACC TGT AGG ATC  (SEQ ID NO: 42)
         G

1830-51  TTCGATACCACCACCTCCACCTTTAC-  (SEQ ID NO: 43)
         CCGGAGACAGGGAGAGGCTCTTCTGC
```

The oligonucleotides 1830-51 and 1830-52 contain an overlap of 24 nucleotides, allowing the two genes to be fused together in the correct reading frame by combining the above PCR products in a third reaction using the outside primers, 1216-52 and 1830-55.

The final PCR gene product (the full length fusion gene) was digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 (see below), also digested with XbaI and BamHI. Ligated DNA was transformed into competent host cells of *E. coli* strain 2596 (GM221, described below). Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysates were analyzed for expression of the fusion via Coomassie stained PAGE gels.

The amino acid sequence of the fusion protein is shown below the corresponding nucleotide sequence:

```
 XbaI
   |
   TCTAGATTTGTTTTAACTAATTAAAGGAGGAATAACATATGGACAAAACTCACACATGTC
 1 ---------+---------+---------+---------+---------+---------+  60
   AGATCTAAACAAAATTGATTAATTTCCTCCTTATTGTATACCTGTTTTGAGTGTGTACAG
                                           M  D  K  T  H  T  C  P

    CACCTTGTCCAGCTCCGGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAAC
 61 ---------+---------+---------+---------+---------+---------+  120
    GTGGAACAGGTCGAGGCCTTGAGGACCCCCCTGGCAGTCAGAAGGAGAAGGGGGGTTTTG
      P  C  P  A  P  E  L  L  G  G  P  S  V  F  L  F  P  P  K  P

     CCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGA
 121 ---------+---------+---------+---------+---------+---------+  180
     GGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCACCACCACCTGCACT
       K  D  T  L  M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S

     GCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATG
 181 ---------+---------+---------+---------+---------+---------+  240
     CGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTAC
       H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A

     CCAAGACAAAGCCCCGGGAGGAGCAGTACAACAGACGTACCGTGTGGTCAGCGTCCTCA
 241 ---------+---------+---------+---------+---------+---------+  300
     GGTTCTGTTTCGGCCCTCCTCGTCATGTTGTCGTGCATGGCACACCAGTCGCAGGAGT
       K  T  K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T

     CCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAG
 301 ---------+---------+---------+---------+---------+---------+  360
     GGCAGGACGTGGTCCTGACCGACTTACCGTTCCTCATGTTCACGTTCCAGAGGTTGTTTC
       V  L  H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N  K  A

     CCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCAC
 361 ---------+---------+---------+---------+---------+---------+  420
     GGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGGTTTCCCGTCGGGGCTCTTGGTG
       L  P  A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E  P  Q

     AGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCT
 421 ---------+---------+---------+---------+---------+---------+  480
     TCCACATGTGGGACGGGGGTAGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGA
       V  Y  T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T  C
```

-continued

```
    GCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGC
481 ---------+---------+---------+---------+---------+---------+  540
    CGGACCAGTTTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCCTCTCGTTACCCGTCG
      L  V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P

    CGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCT
541 ---------+---------+---------+---------+---------+---------+  600
    GCCTCTTGTTGATGTTCTGGTGCGGAGGGCACGACCTGAGGCTGCCGAGGAAGAAGGAGA
      E  N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y

    ACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCG
601 ---------+---------+---------+---------+---------+---------+  660
    TGTCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCCTTGCAGAAGAGTACGAGGC
      S  K  L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V

    TGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTA
661 ---------+---------+---------+---------+---------+---------+  720
    ACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCAT
      M  H  E  A  L  H  N  N  Y  T  Q  K  S  L  S  L  S  P  G  K

    AAGGTGGAGGTGGTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTG
721 ---------+---------+---------+---------+---------+---------+  780
    TTCCACCTCCACCACCATAGCTTCCAGGCTGAGACGCAGTCACCGACCGACGAGCACGAC
      G  G  G  G  G  I  E  G  P  T  L  R  Q  W  L  A  A  R  A  G

    GTGGTGGAGGTGGCGGCGGAGGTATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCAC
781 ---------+---------+---------+---------+---------+---------+  840
    CACCACCTCCACCGCCGCCTCCATAACTCCCGGGTTGGGAAGCGGTTACCGAACGTCGTG
      G  G  G  G  G  G  G  I  E  G  P  T  L  R  Q  W  L  A  A  R

               BamHI
                  |
    GCGCATAATCTCGAGGATCCG
841 ---------+---------+-                                         861
    CGCGTATTAGAGCTCCTAGGC
      A
```

SEQ ID NO: 44 [single strand reading 5'-3' above],
SEQ ID NO: 45 [single strand reading 3'-5' above] and
SEQ ID NO: 46 [the encoded amino acid sequence] pAMG21.

The expression plasmid pAMG21 is available from the ATCC under accession number 98113, which was deposited on Jul. 24, 1996.

GM221 (Amgen Host Strain #2596)

The Amgen host strain #2596 is an *E. coli* K-12 strain that has been modified to contain both the temperature sensitive lambda repressor cI857s7 in the early ebg region and the lacI^Q repressor in the late ebg region (68 minutes). The presence of these two repressor genes allows the use of this host with a variety of expression systems, however both of these repressors are irrelevant to the expression from luxP$_R$. The untransformed host has no antibiotic resistances.

The ribosome binding site of the cI857s7 gene has been modified to include an enhanced RBS. It has been inserted into the ebg operon between nucleotide position 1170 and 1411 as numbered in Genbank accession number M64441 Gb_Ba with deletion of the intervening ebg sequence.

The construct was delivered to the chromosome using a recombinant phage called MMebg-cI857s7 enhanced RBS #4 into F'tet/393. After recombination and resolution only the chromosomal insert described above remains in the cell. It was renamed F'tet/GM101.

F'tet/GM101 was then modified by the delivery of a lacI^Q construct into the ebg operon between nucleotide position 2493 and 2937 as numbered in the Genbank accession number M64441 Gb_Ba with the deletion of the intervening ebg sequence.

The construct was delivered to the chromosome using a recombinant phage called AGebg-LacIQ#5 into F'tet/GM101. After recombination and resolution only the chromosomal insert described above remains in the cell. It was renamed F'tet/GM221. The F'tet episome was cured from the strain using acridine orange at a concentration of 25 ug/ml in LB. The cured strain was identified as tetracyline sensitive and was stored as GM221.

The Fc fusion construct contained in plasmid pAMG21 (referred to herein as pAMG21-Fc-TMP-TMP), which in turn is contained in the host strain GM221 has been deposited at the ATCC under accession number 98957, with a deposit date of Oct. 22, 1998.

Expression. Cultures of pAMG21-Fc-TMP-TMP in *E. coli* GM221 in Luria Broth medium containing 50 μg/ml kanamycin were incubated at 37° C. prior to induction. Induction of Fc-TMP-TMP gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 20 ng/ml and cultures were incubated at 37° C. for a further 3 hours. After 3 hours, the bacterial cultures were examined by microscopy for the presence of inclusion bodies and were then collected by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that the Fc-TMP-TMP was most likely produced in the insoluble fraction in *E. coli*. Cell pellets were lysed directly by resuspension in Laemmli sample buffer containing 10% β-mercaptoethanol and were analyzed by SDS-PAGE. An intense Coomassie stained band of approximately 30 kDa was observed on an SDS-PAGE gel. The expected gene product would be 269 amino acids in length and have an expected molecular weight of about 29.5 kDa. Fermentation was also carried out under standard batch conditions at the 10 L scale, resulting in similar expression levels of the Fc-TMP-TMP to those obtained at bench scale.

Purification of Fc-TMP-TMP.

Cells were broken in water (1/10) by high pressure homogenization (2 passes at 14,000 PSI) and inclusion bodies were harvested by centrifugation (4200 RPM in J-6B for 1 hour). Inclusion bodies were solubilized in 6 M guanidine, 50 mM Tris, 8 mM DTT, pH 8.7 for 1 hour at a 1/10 ratio. The solubilized mixture was diluted 20 times into 2 M urea, 50 mM Tn's, 160 mM arginine, 3 mM cysteine, pH 8.5. The mixture was stirred overnight in the cold. At this point in the procedure the Fc-TMP-TMP monomer subunits dimerize to form the disulfide-linked compound having the structure shown in FIG. **6**C, and then concentrated about 10 fold by ultafiltration. It was then diluted 3 fold with 10 mM Tris, 1.5 M urea, pH 9. The pH of this mixture was then adjusted to pH 5 with acetic acid. The precipitate was removed by centrifugation and the supernatant was loaded onto a SP-Sepharose Fast Flow column equilibrated in 20 mM NaAc, 100 mM NaCl, pH 5(10 mg/ml protein load, room temperature). The protein was eluted off using a 20 column volume gradient in the same buffer ranging from 100 mM NaCl to 500 mM NaCl. The pool from the column was diluted 3 fold and loaded onto a SP-Sepharose HP column in 20 mM NaAc, 150 mM NaCl, pH 5 (10 mg/ml protein load, room temperature). The protein was eluted off using a 20 column volume gradient in the same buffer ranging from 150 mM NaCl to 400 mM NaCl. The peak was pooled and filtered.

III. The following is a summary of in vivo data in mice with various compounds of this invention.

Mice. Normal female BDF1 approximately 10-12 weeks of age.

Bleed schedule. Ten mice per group treated on day 0, two groups started 4 days apart for a total of 20 mice per group. Five mice bled at each time point, mice were bled a minimum of three times a week. Mice were anesthetized with isoflurane and a total volume of 140-160 μl of blood was obtained by puncture of the orbital sinus. Blood was counted on a Technicon HIE blood analyzer running software for murine blood. Parameters measured were white blood cells, red blood cells, hematocrit, hemoglobin, platelets, neutrophils.

Treatments. Mice were either injected subcutaneously for a bolus treatment or implanted with 7 day micro-osmotic pumps for continuous delivery. Subcutaneous injections were delivered in a volume of 0.2 ml. Osmotic pumps were inserted into a subcutaneous incision made in the skin between the scapulae of anesthetized mice. Compounds were diluted in PBS with 0.1% BSA. All experiments included one control group, labeled "carrier" that were treated with this diluent only. The concentration of the test articles in the pumps was adjusted so that the calibrated flow rate from the pumps gave the treatment levels indicated in the graphs.

Compounds. A dose titration of the compound was delivered to mice in 7 day micro-osmotic pumps. Mice were treated with various compounds at a single dose of 100 μg/kg in 7 day osmotic pumps. Some of the same compounds were then given to mice as a single bolus injection.

Activity test results. The results of the activity experiments are shown in FIGS. **4** and **5**. In dose response assays using 7-day micro-osmotic pumps (data not shown) the maximum effect was seen with the compound of SEQ ID NO: 18 was at 100 μg/kg/day; the 10 μg/kg/day dose was about 50% maximally active and 1 μg/kg/day was the lowest dose at which activity could be seen in this assay system. The compound at 10 μg/kg/day dose was about equally active as 100 μg/kg/day unpegylated rHu-MGDF in the same experiment.

IV. Discussion

It is well accepted that MGDF acts in a way similar to human growth hormone (hGH), i.e., one molecule of the protein ligand binds two molecules of the receptor for its activation (Wells, J. A. et al., Ann. Rev. Biochem. 65:609-634 (1996))). This interaction is mimicked by the action of the much smaller TMP peptide. However, the present studies suggest that this mimicry requires the concerted action of two TMP molecules, as covalent dimerization of TMP in either a C-C parallel or C-N sequential fashion increased the in vitro biological potency of the original monomer by a factor of greater than 10^3. The relatively low biopotency of the monomer is probably due to inefficient formation of the noncovalent dimer. A preformed covalent dimer has the ability to eliminate the entropy barrier for the formation of a noncovalent dimer which is exclusively driven by weak, noncovalent interactions between two molecules of the small, 14-residue peptide.

It is interesting to note that most of the tandem dimers are more potent than the C-terminal parallel dimers. Tandem dimerization seems to give the molecule a better fit conformation than does the C-C parallel dimerization. The seemingly unsymmetric feature of a tandem dimer might have brought it closer to the natural ligand which, as an unsymmetric molecule, uses two different sites to bind two identical receptor molecules.

Introduction of the PEG moiety was envisaged to enhance the in vivo activity of the modified peptide by providing it a protection against proteolytic degradation and by slowing down its clearance through renal filtration. It was unexpected that pegylation could further increase the in vitro bioactivity of a tandem dimerized TMP peptide in the cell-based proliferation assay.

V. The following is a summary of in vivo data in monkeys with various compounds of this invention.

In order to evaluate hematological parameters in female rhesus monkeys associated with administration of AMP2 via subcutaneous administration, the following protocol was designed and carried out. Five groups of three monkeys each were assembled. Group 1 served as control and received acetate buffer (20 mM sodium acetate, 0.25 M sodium chloride, pH 5) containing neither AMP2 nor pegylated, recombinant human MGDF (PEG-rHuMGDF). Group 2 received one or more dosage of AMP2 at intervals indicated below; Group 3 received 1000 μg/kg AMP2 at intervals indicated below; Group 4 received 5000 μg/kg AMP2 at intervals indicated below; and Group 5 received 100 μg/kg PEG-rHuMGDF at intervals indicated below.

The day on which the first single dose was administered was designated as Day 0 of Cycle 1. In Cycle 2, doses were administered on Days 21, 23, 25, 28, 30 and 32. During Cycle 3, a single dose was administered on Day 84, and in Cycle 4, a single dose was administration on Day 123. Animals were observed for clinical signs once daily during the acclimation period, three times daily (prior to dosing, immediately to 30 minutes following dosing, and 2 to 3 hours following dosing) on the dosing days, and once daily on the non-dosing days. Food consumption was calculated daily based on the number of food pieces given and the number left over for each animal from 7 days prior to the initiation of the dosing period to the end of the recovery period. Body weight for each animal-was measured twice prior to the dosing regimen and twice during the dosing and recovery periods. Blood samples for hematology were prepared once prior to the initiation of dosing and once on Days 1, 3, 5, 7, 9, 11, 13, 15, 20, 22, 24, 26, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 55, 62, 69, 76, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 111, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 150. For pharmacokinetic analysis. 0.5 ml serum samples were collected once prior to dosing and once at 1, 4 and 24 hours after dosing. Samples were collected on Days 0, 21, 32, 84, and 123 and stored at approximately −70° C. until analysis. For antibody analysis, 2 ml blood samples were collected one week prior to the single dose and once on Days 0 (prior to dosing), 6, 13, 20, 27, 34, 41, 48, 55, 62, 69, 76, 83, 90, 97, 104, 111, 118, 129, 136, 143 and 150. Samples were stored at −70° C. until analysis.

Results indicated that platelet values increased in all treated groups with the largest increases seen in the PEG-rHuMGDF and high dose AMP2 groups. In Cycle 1, peak platelet values increased approximately 3.3-fold and 3.1-fold in the PEG-rHuMGDF group (Day 9) and 5000 μg/kg AMP2 group ((Day 9), respectively, compared to the mean platelet count in the control group. The low dose AMP2 platelet values increased approximately 1.5-fold higher than control on the same specified study days. Similar responses were noted in all other cycles.

However, in Cycle 4, the PEG-rHuMGDF group did not demonstrate as large of an increased platelet count as in the previous cycles. The PEG-rHuMGDF group has increased platelet counts of approximately 2-fold that in the control group 9 days after the dose of this cycle. For comparison, the mean platelet count in the highest dose AMP2 group in Cycle 4 was 3.3-fold higher than the control group. Additionally, PEG-rHuMGDF animals has a mean platelet count 53% lower than the control group mean platelet count at the start of Cycle 4 (per dose) and the mean platelet count for the group at the end of Cycle 4 *(27 days post dose) was 79% lower than that of the control group. For all AMP2 animals, the mean platelet counts at the start and end of Cycle 4 were +15% of the platelet count in the control group.

In Cycle 1 and 2, a trend toward a decrease in red blood cell (RBC) counts was noted in all treated groups as compared to control. The decrease was most evident by Days 41 to 43 and the largest decrease in RBC was noted in the PEG-rHuMGDF group. The counts began returning to normal levels (as compared to control) as early as Day 47. The white blood cell (WBC) levels during Cycles 1 and 2 were dramatically increased (2.6-fold) as compared to control on Day 35. A slight increase was noted in the 5000 μg/kg AMP2 group on Day 33. Values headed toward normal (control) levels beginning on Day 37. A similar response was seen in Cycle 3 with no apparent change in WBC in Cycle 4 in any of the treated groups.

During Cycle 3, RBC counts were slightly decreased by Day 13 (following the single Cycle 3 dose) in all treated groups except for the 500 μg/kg A2 group. RBC values began returning to normal levels (as compared to control) by Day 17.

In Cycle 4, RBC counts decreased in all treated groups as compared to control except in the 500 μg/kg AMP2 group. Unlike the other cycles, there was more than one nadir present in this cycle. These decreases appeared from Day 1-9 post dose and began to recover as early as Day 11.

The results indicated that an increase in platelet counts, above that of control animals could be detected 7 to 9 days following dosing in all treated animals in all cycles tested. It appeared that the repeated dose phase caused a higher response in platelet production as compared to the single dose phases. By Cycle 4, the platelet response elicited by the PEG-rHuMGDF group was lower compared to the previous cycles and compared to that of the high dose AMP2 response. Decreases in RBC counts were noted in Cycles 1, 2, 3 and 4 in most treated groups at some point during each cycle of the study, however, all hematology parameters returned to normal levels (as compared to control) after dosing cessation.

Overall, these results indicated that treatment with AMP2 was well tolerated in the rhesus monkeys and that AMP2 resulted in increased platelet counts after various cycles of treatment. It did not appear, based on the platelet count results, that there was a biologically significant immune-mediated response to AMP2. In contrast, treatment in the various cycles with PEG-rHuMGDF did show an inhibition in platelet response by Cycle 4, suggesting that antibodies to PEG-rHuMGDF have been generated and these anti-MGDF antibodies may be crossreacting with endogenous rhesus TPO.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto, without departing from the spirit and scope of the invention as set forth herein.

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<223> OTHER INFORMATION: Synthetic peptide
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<400> SEQUENCE: 2

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acatgcgtgg tggtggacgt gagccacgaa gaccctgagg tcaagttcaa ctggtacgtg    180

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aaagggcagc cccgagaacc acaggtgtac accctgcccc catcccggga tgagctgacc    420

aagaaccagg tcagcctgac ctgcctggtc aaaggcttct atcccagcga catcgccgtg    480

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tccgacggct ccttcttcct ctacagcaag ctcaccgtgg acaagagcag gtggcagcag    600

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tgtacgcacc accacctgca ctcggtgctt ctgggactcc agttcaagtt gaccatgcac    180

ctgccgcacc tccacgtatt acggttctgt ttcggcgccc tcctcgtcat gttgtcgtgc    240

atggcacacc agtcgcagga gtggcaggac gtggtcctga ccgacttacc gttcctcatg    300

ttcacgttcc agaggttgtt tcgggagggt cgggggtagc tcttttggta gaggtttcgg    360

tttcccgtcg gggctcttgg tgtccacatg tgggacgggg gtagggccct actcgactgg    420

ttcttggtcc agtcggactg gacggaccag tttccgaaga tagggtcgct gtagcggcac    480

ctcaccctct cgttacccgt cggcctcttg ttgatgttct ggtgcggagg gcacgacctg    540

aggctgccga ggaagaagga gatgtcgttc gagtggcacc tgttctcgtc caccgtcgtc    600

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<400> SEQUENCE: 5

Met Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
  1               5                  10                  15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
             20                  25                  30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
         35                  40                  45
```

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
50 55 60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
65 70 75 80

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
85 90 95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
100 105 110

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
115 120 125

Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
130 135 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145 150 155 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
165 170 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
180 185 190

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
195 200 205

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
210 215 220

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225

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 6

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1 5

<210> SEQ ID NO 7
<211> LENGTH: 8
<212> TYPE: PRT
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 7

Gly Gly Gly Asn Gly Ser Gly Gly
1 5

<210> SEQ ID NO 8
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 8

Gly Gly Gly Cys Gly Gly Gly Gly
1 5

<210> SEQ ID NO 9
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 9

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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 10

```
Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Pro
  1               5                  10                  15

Asn Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
             20                  25                  30
```

<210> SEQ ID NO 11
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide; Secondary structure is maintained by disulfide bond between intramolecular Cys residues at positions 9 and 31

<400> SEQUENCE: 11

```
Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu
             20                  25                  30

Ala Ala Arg Ala
         35
```

<210> SEQ ID NO 12
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 12

```
Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Arg Leu Gln Cys Leu
             20                  25                  30

Ala Ala Arg Ala
         35
```

<210> SEQ ID NO 13
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 13

```
Ile Glu Gly Pro Thr Leu Arg Gln Ala Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Ala Leu
```

```
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 14
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 14

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Lys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 15
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Lys residue at position 18 is Bromoacetylated
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 15

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Lys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 16
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 16

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 17
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Lys at position 18 is pegylated
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 17

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Lys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
```

```
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 18
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Cys at position 18 is pegylated
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 18

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 19
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 19

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Asn Gly Ser Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 20
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Monomeric subunit of a homodimer; Subunits in
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      Cys residues at position 18 on each subunit
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 20

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 21
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 21

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15
```

```
Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


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<223> OTHER INFORMATION: Peptide is derivatized at the amino teminus
      with a covalently bonded immunoglobulin Fc region
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 22

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Pro
  1               5                  10                  15

Asn Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
            20                  25                  30


<210> SEQ ID NO 23
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the animo and
      carboxy termini to an immunoglobulin Fc region
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 23

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Pro
  1               5                  10                  15

Asn Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
            20                  25                  30


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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is copvalently bonded at the carboxy
      terminus to an immunoglobulin Fc region
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 24

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 25
<211> LENGTH: 34
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 25
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Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
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Gly Pro Asn Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala
20 25 30

Arg Ala

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<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 26

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 27
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region
<220> FEATURE:
<223> OTHER INFORMATION: Cyclic peptide; Secondary structure is maintained by disulfide linkage between intramolecular Cys residues at positions 9 and 31
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 27

Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 28
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 28

Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu Ala Ala Arg Ala Gly Gly
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Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 29

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<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino
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<400> SEQUENCE: 29

Ile Glu Gly Pro Thr Leu Arg Gln Ala Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Ala Leu
             20                  25                  30

Ala Ala Arg Ala
         35


<210> SEQ ID NO 30
<211> LENGTH: 36
<212> TYPE: PRT
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<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino
      terminus to an immunoglobulin Fc region
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 30

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Lys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
             20                  25                  30

Ala Ala Arg Ala
         35


<210> SEQ ID NO 31
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<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 31

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
             20                  25                  30

Ala Ala Arg Ala
         35


<210> SEQ ID NO 32
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino
      terminus to an immunoglobulin Fc region

<400> SEQUENCE: 32

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15
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Gly Asn Gly Ser Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 33
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is a subunit of a homodimer; Subunits
      in the homodimer are colvantly bonded through a
      disulfide bond between Cys residues at position 18
      of each subunit
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino
      terminus to an immunoglobulin Fc region

<400> SEQUENCE: 33

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
  1               5                  10                  15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
            20                  25                  30

Ala Ala Arg Ala
        35


<210> SEQ ID NO 34
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino
      terminus to an immunoglobulin Fc region

<400> SEQUENCE: 34

Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala
  1               5                  10                  15

Ala Arg Ala Gly Gly Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr
            20                  25                  30

Leu Arg Gln Trp Leu Ala Ala Arg Ala
        35                  40


<210> SEQ ID NO 35
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 35

aaaggtggag gtggtggtat cgaaggtccg actctgcgtc agtggctggc tgctcgtgct     60


<210> SEQ ID NO 36
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 36

acctccacca ccagcacgag cagccagcca ctgacgcaga gtcggacc                  48
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-continued

<210> SEQ ID NO 37
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 37

ggtggtggag gtggcggcgg aggtattgag ggcccaaccc ttcgccaatg gcttgcagca 60

cgcgca 66

<210> SEQ ID NO 38
<211> LENGTH: 76
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 38

aaaaaaagga tcctcgagat tatgcgcgtg ctgcaagcca ttggcgaagg gttgggccct 60

caatacctcc gccgcc 76

<210> SEQ ID NO 39
<211> LENGTH: 126
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 39

aaaggtggag gtggtggtat cgaaggtccg actctgcgtc agtggctggc tgctcgtgct 60

ggtggtggag gtggcggcgg aggtattgag ggcccaaccc ttcgccaatg gcttgcagca 120

cgcgca 126

<210> SEQ ID NO 40
<211> LENGTH: 124
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 40

ccaggctgag acgcagtcac cgaccgacga gcacgaccac cacctccacc gccgcctcca 60

taactcccgg gttgggaagc ggttaccgaa cgtcgtgcgc gtattagagc tcctaggaaa 120

aaaa 124

<210> SEQ ID NO 41
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 41

Lys Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
1 5 10 15

Ala Ala Arg Ala Gly Gly Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro
20 25 30

Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
35 40

<210> SEQ ID NO 42

<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 42

aacataagta cctgtaggat cg 22

<210> SEQ ID NO 43
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 43

ttcgatacca ccacctccac ctttacccgg agacagggag aggctcttct gc 52

<210> SEQ ID NO 44
<211> LENGTH: 861
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

tctagatttg ttttaactaa ttaaaggagg aataacatat ggacaaaact cacacatgtc 60

caccttgtcc agctccggaa ctcctggggg gaccgtcagt cttcctcttc cccccaaaac 120

ccaaggacac cctcatgatc tcccggaccc ctgaggtcac atgcgtggtg gtggacgtga 180

gccacgaaga ccctgaggtc aagttcaact ggtacgtgga cggcgtggag gtgcataatg 240

ccaagacaaa gccgcgggag gagcagtaca acagcacgta ccgtgtggtc agcgtcctca 300

ccgtcctgca ccaggactgg ctgaatggca aggagtacaa gtgcaaggtc tccaacaaag 360

ccctcccagc ccccatcgag aaaaccatct ccaaagccaa agggcagccc cgagaaccac 420

aggtgtacac cctgccccca tcccgggatg agctgaccaa gaaccaggtc agcctgacct 480

gcctggtcaa aggcttctat cccagcgaca tcgccgtgga gtgggagagc aatgggcagc 540

cggagaacaa ctacaagacc acgcctcccg tgctggactc cgacggctcc ttcttcctct 600

acagcaagct caccgtggac aagagcaggt ggcagcaggg gaacgtcttc tcatgctccg 660

tgatgcatga ggctctgcac aaccactaca cgcagaagag cctctccctg tctccgggta 720

aaggtggagg tggtggtatc gaaggtccga ctctgcgtca gtggctggct gctcgtgctg 780

gtggtggagg tggcggcgga ggtattgagg gcccaaccct tcgccaatgg cttgcagcac 840

gcgcataatc tcgaggatcc g 861

<210> SEQ ID NO 45
<211> LENGTH: 861
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 45

agatctaaac aaaattgatt aatttcctcc ttattgtata cctgttttga gtgtgtacag 60

gtggaacagg tcgaggcctt gaggaccccc ctggcagtca gaaggagaag gggggttttg 120

ggttcctgtg ggagtactag agggcctggg gactccagtg tacgcaccac cacctgcact 180

cggtgcttct gggactccag ttcaagttga ccatgcacct gccgcacctc cacgtattac 240

ggttctgttt cggcgccctc ctcgtcatgt tgtcgtgcat ggcacaccag tcgcaggagt 300

ggcaggacgt ggtcctgacc gacttaccgt tcctcatgtt cacgttccag aggttgtttc 360

```
gggagggtcg ggggtagctc ttttggtaga ggtttcggtt tcccgtcggg gctcttggtg    420

tccacatgtg ggacgggggt agggccctac tcgactggtt cttggtccag tcggactgga    480

cggaccagtt tccgaagata gggtcgctgt agcggcacct caccctctcg ttacccgtcg    540

gcctcttgtt gatgttctgg tgcggagggc acgacctgag gctgccgagg aagaaggaga    600

tgtcgttcga gtggcacctg ttctcgtcca ccgtcgtccc cttgcagaag agtacgaggc    660

actacgtact ccgagacgtg ttggtgatgt gcgtcttctc ggagagggac agaggcccat    720

ttccacctcc accaccatag cttccaggct gagacgcagt caccgaccga cgagcacgac    780

caccacctcc accgccgcct ccataactcc cgggttggga agcggttacc gaacgtcgtg    840

cgcgtattag agctcctagg c                                              861


<210> SEQ ID NO 46
<211> LENGTH: 269
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 46

Met Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
  1               5                  10                  15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
             20                  25                  30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
         35                  40                  45

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
     50                  55                  60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
 65                  70                  75                  80

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
                 85                  90                  95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
            100                 105                 110

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
        115                 120                 125

Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
    130                 135                 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145                 150                 155                 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                165                 170                 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
            180                 185                 190

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
        195                 200                 205

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    210                 215                 220

Ser Pro Gly Lys Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg
225                 230                 235                 240

Gln Trp Leu Ala Ala Arg Ala Gly Gly Gly Gly Gly Gly Gly Gly Ile
                245                 250                 255

Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
            260                 265
```

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A compound that binds to an mpl receptor comprising the structure

$TMP_1\text{-}(L_1)_n\text{-}TMP_2$

wherein the C-terminus of the TMP_1 peptide is linked to the N-terminus of the TMP_2 peptide, optionally via L_1,

wherein TMP_1 and TMP_2 are each independently selected from the group of core compounds comprising the structure selected from the group consisting of:

a) $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

b) $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

C) $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

d) $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

e) $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

f) $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln and Asn;

X_9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

g) $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Tyr, Cys, Ala, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys; and

h) $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X_{10} is selected from the group consisting of Leu, Val, Ala, Phe, Met, and Lys; and

wherein L_1 is a linker; and

n is 0 or 1, wherein when n is 1, L_1 is independently selected from the linker groups consisting of Y_n, wherein Y is a naturally occurring amino acid or a stereoisomer thereof and n is 1 through 20;

and physiologically acceptable salts thereof.

2. The compound according to claim **1** wherein said TMP_1 and TMP_2 are independently selected from the group consisting of:

X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-;
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}; and
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9- X_{10}-X_{11}-X_{12}-X_{13}-X_{14},

wherein X_2-X_{10} are as defined and X_1 and X_{11}-X_{14} are selected from the group consisting of:

a) X_1 is selected from the group consisting of Ile, Val, Leu, Ser, and Arg;

X_{11} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Ser, Thr, Lys, His, and Glu;

X_{12} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Gly, Ser, and Gln;

X_{13} is selected from the group consisting of Arg, Lys, Thr, Val, Asn, Gln, and Gly; and

X_{14} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Thr, Arg, Glu, and Gly;

b) X_1 is selected from the group consisting of Ile, Ala, Val, Leu, Ser, and Arg;

X_{11} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Thr, Lys, His, and Glu;

X_{12} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Gly, Ser, and Gln;

X_{13} is selected from the group consisting of Arg, Lys, Thr, Val, Asn, Gln, and Gly; and

X_{14} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Thr, Arg, Glu, and Gly; and

c) X_1 is selected from the group consisting of Ile, Ala, Val, Leu, Ser, and Arg;

X_{11} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Ser, Thr, Lys, His, and Glu;

X_{12} is selected from the group consisting of Ala, Ile, Val, Leu, Gly, Ser, and Gln;

X_{13} is selected from the group consisting of Arg, Lys, Thr, Val, Asn, Gln, and Gly; and

X_{14} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Thr, Arg, Glu, and Gly.

3. The compound according to claim **1** wherein said TMP_1 and/or TMP_2 are derivatized as set forth in one or more of the following:

one or more of the peptidyl [—C(O)NR—] linkages (bonds) have been replaced by a non-peptidyl linkage selected from the group consisting of: a —CH_2-carbamate linkage [—CH_2—OC(O)NR—]; a phosphonate linkage; a —CH_2-sulfonamide [—CH_2—$S(O)_2NR$—] linkage; a urea [—NHC(O)NH—] linkage; a —CH_2-secondary amine linkage; and an alkylated peptidyl linkage [—C(O)NR^6— where R^6 is lower alkyl];

the N-terminus is a —NRR^1 group; to a —NRC(O)R group; to a —NRC(O)OR group; to a —$NRS(O)_2R$ group; to a —NHC(O)NHR group where R and R^1 are hydrogen and lower alkyl with the proviso that R and R^1 are not both hydrogen; to a succinimide group; to a benzyloxycarbonyl-NH— (CBZ—NH—) group; or to a benzyloxycarbonyl-NH— group having from 1 to 3 substituents on the phenyl ring selected from the group consisting of lower alkyl, lower alkoxy, chloro, and bromo;

the C terminus is —C(O)R^2 where R^2 is selected from the group consisting of lower alkoxy and —NR^3R^4 where R^3 and R^4 are independently selected from the group consisting of hydrogen and lower alkyl.

4. The compound according to claim **1** wherein all of the amino acids have a D configuration.

5. The compound according to claim **1** wherein at least one of the amino acids has a D configuration.

6. The compound according to claim **1** wherein L_1 comprises ((Gly)$_n$, wherein n is 1 through 20, and when n is greater than 1, up to half of the Gly residues may be substituted by another amino acid selected from the remaining 19 natural amino acids or a stereoisomer thereof.

7. The compound according to claim **1** wherein L_1 is selected from the group consisting of

$(Gly)_3Lys(Gly)_4$;	(SEQ ID NO: 6)
$(Gly)_3AsnGlySer(Gly)_2$;	(SEQ ID NO: 7)
$(Gly)_3Cys(Gly)_4$;	(SEQ ID NO: 8) and
GlyProAsnGly.	(SEQ ID NO: 9)

8. The compound according to claim **1** wherein L_1 comprises a Cys residue.

* * * * *